STUDIES ON THE URINARY AND TROPHOBLASTIC CHORIONIC GONADOTROPINS FROM PATIENTS WITH HYDATIDIFORM MOLE

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LIST OF ABBREVIATIONS

FSH	Follicle-stimulating hormone.
HCG	Human chorionic gonadotropin.
IgG	γ-immunoglobulin.
IU	International unit of HCG activity, measured against the second international reference preparation of HCG.
LH	Luteinizing hormone.
MT-HCG	HCG from molar tissue.
MU-HCG	HCG from molar urine.
PBS	0.01M phosphate buffer, pH 7.3. 0.9% NaCl.
SDS	Sodium dodecyl sulphate.
TCA	Trichloroacetate
U-HCG	HCG from first trimester pregnant urine.

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INTRODUCTION

Hydatidiform mole is a placental disorder which arises in early pregnancy. The chorionic villi degenerate and become enlarged into fluid-filled, transparent and grape-like clusters due to the persistent normal activity of immature trophoblast in the absence of a functioning chorionic circulation (Hertig, 1968).

The incidence of hydatidiform mole is geographically dependent. Its occurence in Asian and Western African countries and Mexico is common, but is relatively rare in Europe and in North America. The incidence is 1:242 deliveries in Hong Kong (Chun et al., 1964) and 1:173 in Philippine (Acosta-Sison and Panlilio, 1951) as compared with 1:2,000 in England (Jeffcoate, 1957) and 1:2,500 in United States (Novak, 1962). On a more detailed and statistical observation, the epidemiology of hydatidiform mole associated with race, malnutrition, age, socio-economic level, climate and congenital factors have been noted (MacGregor et al., 1969; Teoh et al., 1971; Matalon et el., 1972; McCorriston, 1968; Reynolds, 1976; Poen and Djojopranoto, 1965; De George, 1970).

The etiology of hydatidiform mole remains unknown and its genesis is still uncertain. Several theories have been proposed to explain the pathogenesis of hydatidiform mole. According to Hertig (1968), the probable sequence

of events in the development of mole are:

a. the early death or absence of the embryo,

b. a disappearance of villious blood vessels,

c. the accumulation of stromal fluid through the activity of a still functioning trophoblast, and

d. the ultimate globular swelling of the branch(es) of the involved villus.

Hertig and Edmonds (1940) stated that the trophoblastic hyperplasia at the surfaces of molar villus is variable, and this is thought to be the consequence of "stretching", so that enlargement of the villus-vesicle necessitates such epithelial activity in order to maintain the integrity of its surface.

According to the hypothesis of Park (1967), the primary disturbance lies in the trophoblast. This is functionally and structurally abnormal with the excess absorption of fluid into the villi, destruction of blood vessels and death of the embryo. Whether the trophoblastic abnormality is most probably to be regarded as hyperplasia, dysplasia or benign neoplasia is still not clear.

Reynolds (1976), studying the early placental morphogensis and the geographical distribution of hydatidiform mole, related this placental disorder to nutritional deficiencies. Since nucleic acid synthesis, so essential for embryonic and trophoblastic growth on a rapid and critical time basis for normal development, is dependent

on the presence of folic acid and amino acids. When these specific diets are lacking at a time of high need, embryo death, abnormality, and/or avascularity of trophoblastic placental villi may be the earliest pathogenic sign of hydatidiform mole. Cytogenetic studies also showed that the initial step in the formation of a mole is related to metabolic disturbances of the trophoblastic tissue secondary to abnormal pregnancy and a neoplasmic continum exists as evidenced by the gradual change in chromosome number and nuclear DNA content of the molar trophoblast (Ferenczy and Richart, 1973; Vassilako et al., 1977).

On the other hand, the isolation of virus from hydatidiform mole has given some evidence for a viral etiology (Okudaira and Stranss, 1967; Park, 1971) and further work is still being undertaken to verify these findings.

Choriocarcinoma is closely related to hydatidiform mole. Women who have hydatidiform mole are considered as a high-risk group in developing choriocarcinoma with report ranging from 2-20% (Kolstad et al., 1965; Schiffer et al., 1960) or 1,000-4,000 times higher than normal pregnancy (Teoh et al., 1972). And 40-50% of choriocarcinoma cases have been found to have mole history (Matalon et al., 1972; Ringertz, 1970). In countries with a high incidence of hydatidiform mole, the risk of a hydatidiform mole patient to develop choriocarcinoma is also high. But this extent has declined, it most probably reflects the

earlier and better recognition and therapy for hydatidiform mole.

Most commonly, a presumptive diagnosis is made on the basis of the presenting clinical signs together with the demonstration of significantly elevated human chorionic gonadotropin (HCG) excretion which is greater than that expected for normal intrauterine pregnancy at the same gestational age (Curry et al., 1975). However, HCG levels are not always high in molar pregnancies. By measuring the immunoactivity of 24 hour urinary HCG from 111 patients with a diagnosis of hydatidiform mole, MacGregor et al. (1969) reported that 14 patients were below 25,000IU, 46 patients between 25,000-250,000IU, 60 patients between 250,000-500,000IU, and 35 patients above 500,000IU.

HCG is a glycoprotein hormone, synthesized by the syncytiotrophoblast (Dreskin et al., 1970) and demonstrable in serum by 8 to 12 days after conception. Since the discovery of the presence of this hormone in the urine of pregnant women by Aschheim and Zondek in 1927, estimation of its activity by various means has been the basis for diagnostic tests of early pregnancy. Its actions include maintenance of corpus luteum of pregnancy, stimulation of testosterone release by fetal testes, and possible effects on steroidogenic activity of the fetal adrenal and of the placenta (Canfield et al., 1971). During pregnancy, the urine level of HCG rises continuously to a peak of 30,000-

50,000IU/24 hours during the first 10-12 weeks; thereafter it declines to maintain a lower level in the last half of the pregnancy (Canfield et al., 1971). While in patients with gestational trophoblastic diseases such as hydatidiform mole, chorioadenoma and choriocarcinoma, an elevated level of urinary HCG may occasionally exceed 1,000,000IU/24 hours (Canfield and Morgan, 1973). In addition to its trophoblastic origin, HCG secretion has been associated with tumours of nonendocrine origin. Tumours of gastrointestinal tract are found to have the highest incidence of ectopic production of HCG. As a group, tumours of the stomach, liver, and pancreas have the highest association with ectopic HCG secretion. In some cases, the level of HCG secreted by tumours of the stomach exceeds that secreted in the first trimester of pregnancy (Vaitukaitis et al., 1976). Quite interestingly, patients with a wide variety of gastrointestinal tract abnormalities such as regional enteritis, chronic ulcerative colitis, cirrhosis, gastric ulcer and duodenal ulcer, HCG may also be found circulating in peripheral blood (Vaitukaitis et al., 1976).

Early in 1960, highly purified HCG from the urine of pregnant women with biological potency of 12,000IU/mg were obtained by Got and Bourrillon (1960). Starting from commercial available U-HCG with biological potencies of 1,500-3,000IU/mg, a number of authors obtained products with potencies of 12,000 to 20,000IU/mg (Bahl, 1969a; Van

Hell, 1968 & 1974; Bell et al., 1969; Mori, 1970; Brossmer et al., 1971; Ashitaka et al., 1970; Graesslin et al., 1972; Canfield et al., 1971; Qazi et al., 1974; Okumura et al., 1973). Their purification methods employed several chromatographic steps or isoelectric focusing in addition to ion exchange chromatography and/or gel filtration.

HCG originated from trophoblastic diseases were also studied by several workers. Reisfeld and Hertz (1960) succeeded in preparing highly purified HCG with biological potencies 10,000-12,000IU/mg from the urine of patients with trophoblastic tumours, including choriocarcinoma, hydatidiform mole, chorioadenoma destruens, and syncytial endometritis. Their isolation procedure involves adsorption on kaolin, DEAE-cellulose chromatography and adsorption chromatography on $BaCO_3$. Ashitaka et al. (1972) obtained two highly active fractions (LH-like and FSH-like) with biological potencies of 23,000 and 8,6000IU/mg from the chorionic tissue of patients with hydatidiform mole by percolation, Sephadex gel filtration, CM-C, DEAE-cellulose and DEAE-Sephadex chromatography. Pala et al.(1973) obtained, by DEAE-cellulose, gel filtration on Sephadex G-100 and DEAE-Sephadex, purified HCG fractions from urine, peripheral plasma and chorionic tissue of a woman with hydatidiform with biological potencies of 20,000, 23,000 and 6,500IU/mg, respectively. Chan (1973) and Chan et al. (1974) of this laboratory, using salt precipitation and DEAE-Sephadex ion

exchange chromatography in conjunction with Sephadex G-200 and Sephadex G-100 gel filtration, obtained highly purified HCG from molar tissue with an immunological potency of 21,000IU/mg.

Among the various sources, HCG from the first trimester of pregnant women has been extensively studied, because of its easy availability. The amino acid composition of U-HCG shows an unusual high content of proline, a fairly large amount of serine but the absence of tryptophan. This is markedly similar to that of human luteinizing hormone (hLH) with slightly differences in histidine, serine and proline content (Bahl, 1973). Structurally, proline prohibits the formation of α-helix in its immediate vicinity along the peptide chain, and one might expect that this would indicate a low helical content. On the basis of circular dichroism studies, Mori and Hollands (1971) and Hilgenfeldt et al. (1972) confirmed the absence of the α-helical conformation in HCG.

Like other gonadotropins - luteinizing hormone (LH) and follicle-stimulating hormone (FSH),

HCG has two nonidentical and noncovalently linked subunits, designated α and β. These subunits can be dissociated by incubation with 8 M urea and then readily isolated by ion exchange chromatography (Swaminathan and Bahl, 1970; Morgan and Canfield, 1971). The isolated subunits possess essentially none of the native biological

activity, but can be recombined with substantial restorating of this activity (Morgan et al., 1974).

The amino acid sequences of both α and β subunits of HCG have been determined by two groups of authors. There is an essential agreement between the two proposals (Bellisario et al., 1973; Morgan et al., 1975) for the α primary structure. The total number of amino acid in this subunit varies from 89 to 92. Morgan et al. (1975) found that approximately 10 and 30% of the α chains lack the initial 2 and 3 N-terminal residues respectively. Oligosaccharide chains are attached to asparagine at 52 and 78, and the amino acid sequences in these positions are of the type Asn-X-Thr which is commonly associated with carbohydrate attachments. The calculated molecular weight is about 14,900 with 10,200 for protein and 4,700 for carbohydrate. HCG-α is closely related to the α subunits of other glycoprotein hormones and is probably identical with the hLH-α (Sairam et al. 1972) except that the proposal for hLH-α lacks the 3 N-terminal residues of HCG-α.

There are significant discrepancies between these authors for the primary structure of HCG-β. According to Carlsen et al. (1973), this peptide consists of 147 amino acid residues, two oligosaccharide chains linked to asparagine residues 13 and 30 and three linked to serine residues 118, 129 and 131. The molecular weight is estimated to be 23,000, approximately 16,000 for the protein portion

and 7,000 for the carbohydrate. However, the result of Morgan et al. (1975) shows HCG have only 145 amino acid residues, oligosaccharide side chains are found at residue 13, 30, 121, 127, 132 and 138 where the last four are linked to the serine residues. In addition, the amino acid sequence is different, especially at the COOH-terminus. The HCG-β sequence bears a marked homology with hLH-β which possesses 115 residues of which approximately 80% are identical with those of HCG-β when the protein structures are aligned from the N-terminals. HCG-β thus possesses an additional 30 residues at the COOH-terminus not found in hLH-β nor any other glycoprotein hormone-β subunits (Closset et al., 1973; Shome & Parlow, 1974).

It is quite interesting that antisera generated against intact HCG and HCG-α have been shown to react with HCG-α, HCG and hLH (Donini et al., 1975), while anti-HCG-β antiserum only reacts with intact HCG and HCG-β, but not LH (Ashitaka et al., 1974a). The specificity of this anti-HCG-β antiserum, which is used to measure selectively HCG in samples containing both LH and HCG, can be probably accounted by the significant COOH-terminal differences between the subunits of LH and HCG. In contrast to their immunological behaviors, LH and HCG have essentially indistinguishable biological activity in all conventional bioassay systems. This imples that the COOH-terminal portion of HCG-β is not a requisite for biological activity, and indeed it was

confirmed by Louvet et al. (1974) who prepared antiserum to COOH-terminal portion of HCG-β, which bound HCG with affinity, failed to neutralize the biological activity of HCG.

U-HCG contains quite a large amount of carbohydrates, approximately 25 to 31% have been reported (Got et al., 1960; Goverde et al., 1968; Bahl, 1969a; Mori, 1970; Canfield et al., 1971). The carbohydrate components are L-fucose, D-galactose, D-mannose, N-acetylglucosamine and N-acetylgalactosamine. The oligosaccharide side chains are linked to the peptide backbone by either N-acetylglucosamyl-asparagine or N-acetylgalactosamyl-serine linkages. The monosaccharide compositions of HCG-α and HCG-β show marked differences. The major difference is reflected as the presence of N-acetylgalactosamine and fucose in HCG-β and their virtually absence from HCG-α. In addition, the amounts of sialic acid and galactose are higher in HCG-β than in HCG-α.

Up to now, there is still little information on the structure of carbohydrate units in HCG. In 1969, Bahl (1969b) reported his studies on the nature of the oligosaccharide units in HCG. Using specific glycosidases such as neuraminidase, β-galactosidase, β-N-acetylglucosaminidase, α-mannosidase and α-L-fucosidase to cleave stepwisely the oligosaccharide chain in the digestive fragments of HCG, he suggested HCG contains two main asparagine-linked hetero-

saccharide units which are complex and bulky. In addition, three small heterosaccharide units composed of NANA-Gal-GalNAc-Ser were identified on the basis of serine loss after alkaline degradation of HCG. However, Bahl's later communication (Bahl et al., 1972) accounted for a total of seven carbohydrate units instead of five as previously reported. In the light of more recent data (Morgan et al., 1975) which account for a total of eight carbohydrate units, Bahl's previous results have to be revised.

The role of carbohydrate in HCG related to their biological and immunological behaviour has been studied by several investigators. The removal of sialic acid residues from HCG reduces markedly its biological activity as estimated by the ventral prostate weight assay or ovarian ascrobic acid depletion assay (Van Hall et al., 1971a). Van Hall et al. (1971b), relating the percent desialylation to the residual biological activity of the desialylated hormonal preparations, found a progressive decrease in both biological and in plasma half-life of the hormone. Although the biological activity of HCG markedly decreases after desialylation, however, the removal of the exocyclic carbon atoms (C8 and C9) of sialic acid does not markedly reduce its biological activity (Vaitukaitis et al., 1971a,b). On the other hand, desialylated HCG is as potent as fully sialylated HCG when tested in vitro using either rat testis Leydig cells for binding to the plasma membrane

or stimulation of testosterone synthesis from Leydig cells (Bahl and März, 1974; Moyle et al., 1975). This significant disparity in biological activity of the hormone when assayed in vivo and in vitro has been accounted for by its markedly altered plasma half-life (Vaitukaitis et al., 1971a,c). The penultimate sugar residue of the HCG carbohydrate side chain when exposed after desialization is galactose (Bahl, 1969b). This galactosyl residue has been confirmed (Morell et al., 1971; Montreuil, 1975) to result in a more avid hepatic uptake of the desialylated glycoprotein hormone and a rapid disappearance of that form from the plasma of test animals. Other desialylated hormonal glycoproteins, except transferrin, behave similarly (Morell et al., 1971).

In contrast to the biological activity of HCG which is markedly affected by desialylation, its immunological activity is unchanged (Van Hall et al., 1971a; Bahl and März, 1974) as determined by radioimmunoassay. Bahl and März (1974), measuring the immunological activities of HCG derivatives lacking various sugar residues, found that a slight decrease in the activity was observed with the cleavage of each of the monosaccharide. This may be due to the change of the outer structure of the molecule and consequently affects the affinity of the antibodies for these molecules. They also suggested that the carbohydrate part of the molecule particularly the sialic acid, galactose,

N-acetylglucosamine and mannose residues did not act as antigenic determinants.

The role of carbohydrate part of HCG in the mechanism of hormone action was investigated by measuring the ability of HCG derivatives which are lacking in various sugar residues by sequential treatment with neuraminidase, β-D-galactosidase, β-D-N-acetylglucosaminidase and α-D-mannosidase to bind to rat Leydig cells and stimulate them to synthesize testosterone and cyclic AMP (Bahl and März, 1974; Moyle et al., 1975). All the glycosidase-treated HCG derivatives were found to stimulate steroidogenesis. Sequential removal of sialic acid, galactose, N-acetylglucosamine, and mannose led to a progressive loss in potency of HCG, however, progressive increase in the doses of the hormone derivatives was capable of eliciting the same maximal steroidogenic response as HCG. When the glycosidase-treated HCG derivatives were examined for their ability to stimulate cyclic AMP accumulation, only the asialo derivatives were capable of producing significant cyclic AMP accumulation. The remainder rarely elicited more than 10% of the response seen even after treatment with large amount of HCG. This observation showed that the HCG derivatives were potent inhibitors of HCG-induced cyclic AMP accumulation and that removal of carbohydrates did not interfere with the affinity of the hormone derivatives for the cell. As a matter of fact, removal of sialic acid and galactose increased the

ability of the hormone to bind to Leydig cells, whereas removal of N-acetylglucosamine and mannose decreased this binding property slightly. Based on the above observations and the studies on LH in which the carbohydrate content is much lower than that of HCG but stimulates the cyclic AMP accumulation and steroidogenesis to the same maximal values as HCG, these authors (Moyer et al., 1975: Bahl and März, 1974) suggested that the carbohydrates were not involved directly in binding. It was possible that the carbohydrate brought about the activation of adenyl cyclase by interactions with the enzyme directly or through the membrane implying a close proximity of the binding and the adenyl cyclase sites. Alternatively, the carbohydrate might be required to maintain the proper conformation of the hormone or the hormone receptor complex necessary for enzyme activation. Two functionally distinct receptors in Leydig cells might exist, one mediating steroidogenesis, while the other mediatingcyclic AMP accumulation. On the other hand, one may also predict that besides its binding site HCG has two active sites, one for steroidogenesis and the other for cyclic AMP accumulation.

An immunosuppressive action of HCG has been inferred from the complete inhibition of the response of lymphocytes to phytohemagglutinin by HCG (Contractor and Davies, 1973; Adcock et al., 1973). These observations support the theory

that fetus with antigens at least 50% different from that of the mother is accepted because HCG represents trophoblastic surface antigen and blocks the action of maternal lymphocytes. However, recently, Merz et al. (1976) and Pattilo et al. (1976) found that purified HCG did not inhibit the phytohemagglutinin induced tranformation lymphocytes in the mixed lymphocyte reaction. On the contrary, non-HCG substances isolated from crude HCG were found to inhibit the transformation of lymphocytes (Merz et al., 1976; Pattilo et al., 1976).

The physicochemical, immunological and biological studies mentioned above were carried out with U-HCG. HCG from trophoblastic diseases have also been studied. Reisfeld and Hertz (1960) were the first to report the differences in chemical structure of HCG from normal pregnancy and choriocarcinoma. Lewis et al. (1964) demonstrated that no antigenic differences were apparent between these two HCGs. Canfield et al. (1971) indicated that HCG secreted by patients with choriocarcinoma had the same amino acid composition as pregnancy HCG, and the differences in chemical properties might be attributed to variations in the carbohydrate structure of choriocarcinoma HCG. Ashitaka et al. (1972), characterizing their purified HCGs, found that HCG from hydatidiform mole contained much more sialic acid and hexose, but much less hexosamine than HCG from normal trophoblastic tissue and urine, and they also suggested that the protein

cores of HCGs of different origins did not appear to differ. By means of polyacrylamide gel electrophoresis, Pala et al. (1973) observed no differences in the molecular weight of purified HCG fractions obtained from urine, plasma and chorionic tissue of hydatidiform mole. Although U-HCG has been found to have blood-group A-like activity as shown by the hemagglutination inhibition technique (März and Bahl, 1973), Fung (1975) of this laboratory did not observe this property in molar HCG. This discrepancy is thought to be the differences in carbohydrate structure. Studing the plasma, urine and tissue extracts of patients with HCG secreting tumours and gestational trophoblastic diseases, Vaitukaitis (1973) indicated the presence of altered forms of HCG with higher or lower molecular weight by chromatography on a calibrated Sephadex G-100 column. Since altered forms of HCG were present in urine, plasma and tissue, these HCGs were not an artifact of excretion, but arised during synthesis.

In normal pregnancy, chorionic tissue was found to have large amount of free HCG-β in all trimesters of pregnancy (Vaitukaitis, 1974; Ashitaka et al., 1974b). With the progression of gestation, there is a marked excess of HCG-α in sera, and free HCG-β is absent in either chorionic tissue and in sera during pregnancy (Vaitukaitis, 1974). Based on these findings, its has been postulated that the control of placental HCG secretion probably resides on the

control of synthesis HCG-β (Vaitukaitis et al., 1976).

Recently, tissue extracts, blood and urine of women with gestational trophoblastic diseases were analyzed for HCG and its subunits by Vaitukaitus and her coworkers (Vaitukaitis et al., 1976; Vaitukaitis and Ebersole, 1976). Extracts of four hydatidiform moles contained primarily HCG but no α-subunit of that hormone. One of the molar extracts contained a small amount of free HCG-β not observed in the extracts of the other three moles. Plasma and urine samples from 18 women with localized or metastatic gestational trophoblastic disease contained HCG but no free α or β subunits. Quite surprisingly, the neoplasm of all these patients readily responded to chemotherapy and had no evidence of disease for at least one year. On the other hand, three women with widely metastatic tumours died in spite of extensive chemotherapy. Tumour extracts, plasma, or urine of these patients contained HCG in addition to large amount of free HCG-α or HCG-β or both. These results clearly show a disparity between the forms of HCG found in normal pregnancy and those found in the neoplastic trophoblast with varying degrees of anaplasia. In addition, varying quantities of HCG, HCG-α, and HCG-β were present in blood, urine or tumour tissue extracts of patients with nongestational choriocarcinoma and of patients with tumours ectopically secreting HCG. Their tumours were usually found to be fatal. In clinical aspect, these findings have been

adsorption, of a biological macromolecule from its crude preparation on an affinity adsorbent which is prepared by the covalent immobilization of a specific ligand on a solid polymeric matrix. The contaminating proteins are removed unretarded. The desorption of macromolecule from the affinity column is achieved either by perturbing the interaction between the macromolecule and the adsorbent or by including a competing ligand in the eluting buffer. For obvious reasons, the design of a new affinity chromatographic system for a given macromolecule requires individual attention in the selection and attachment of the ligand as well as selection of the buffer conditions for the adsorption and desorption processes. Detailed descriptions on the principles and applications of affinity chromatography have been reviewed by a number of authors including O'Carra (1974), Dean and Harvey (1974), Parikh and Cuatrecasas (1975) and Yuan et al. (1975).

In immunoaffinity chromatography, the specific reactions of antibodies with antigens are used as the basis. Assoociation constants range from 10^5 to 10^8 (Eisen and Siskind, 1964). The introduction of agarose activated with CNBr (Axen et al., 1967) facilitated the preparation of immunoadsorbents with higher capacity and superior physical properties. This method has been used to purify antibodies against polypeptide hormones, enzymes, glycosides. Conversely, antibodies immobilized on solid supports have been used to purify poly-

peptide hormones, enzymes, membrane proteins, viruses and labelled peptides derived from modified proteins (Murphy, 1974; Yuan et al., 1975). Wong (1976) of this laboratory, using a single immunoaffinity chromatographic step, achieved a 57-fold purification of HCG from term placenta. In the present studies, Wong's method was adopted.

MATERIALS AND METHODS

Materials:

Analytical grade reagents were used in all experiments. U-HCG (Lot no. CG-10), synthetic N-acetyl-neuraminic acid and dansyl amino acid standards were obtained from Sigma Chemical Company (U.S.A.). The polyamide sheet (15 x 15 cm) used for N-terminal amino acid analysis was a product of Cheng-Chin Trading Company (Taiwan). The protein markers used in molecular weight determination were obtained from Schwarz/Mann Chemical Company (U.S.A.). HCG immunoactivity was measured using the latex agglutination inhibition test kit supplied by Organon Inc. (Holland) under the trade name of Pregnosticon Planotest. For gas liquid chromatography, the column packing materials, 3% Poly A-103 on 100/120 mesh Gas Chrom. Q and 3% ECNSS-M on 100/120 mesh Gas Chrom. Q_3 were obtained from Applied Science Laboratories (U.S.A.).

White albino rabbits used for antiserum production were three months old and from a local source.

Clinical materials were obtained from Queen Elizabeth Hospital. Molar tissue was collected and immediately delivered to the laboratory at the time of vaginal removal or hysterectomy. 24 hour urine was collected before operation.

PART I. PURIFICATION OF HCG FROM TROPHOBLASTIC TISSUE AND URINE OF PATIENTS WITH HYDATIDIFORM MOLE

Through out the following fractionation procedures, the HCG activity and the protein content of the samples were continuously monitored. Hormonal activity was measured by latex agglutination inhibition test with a serial diluent of 0.01M phosphate buffer, pH 8.0, containing 1% bovine serum albumin and 0.9% NaCl. Protein content was determined by absorption at 280nm and by Folin-Ciocalteau method of Lowry et al. (1951) using bovine serum albumin as standard. HCG specific activity was expressed in IU/mg protein.

1. Fractionation by Salt and Acid Precipitation:

All procedures were performed at $4^{o}C$ in a cold room or in a cooling apparatus.

a) Molar Tissue (MT).

Trophoblastic tissue was carefully washed with 0.9% NaCl to remove blood. About 70 gm of the washed tissue was homogenized for 3 x 1 minute blending in 400 ml 0.15 M NH_4HCO_3 using a Waring blender. The homogenate was adjusted to pH 4 with 1 N HCl and stirred for 2 hours. After removal of the precipitate by centrifugation at 10,000 x g for 20 minutes, the super-

natant was adjusted to pH 3 with 0.5 M H_3PO_4. The mixture was then centrifuged at 10,000 x g for 20 minutes to remove precipitate and $(NH_4)_2SO_4$ was added to the supernatant until 30% saturation had been reached. After stirring overnight, the precipitate was removed by centrifugation at 10,000 x g for 20 minutes and then the salt concentration of the supernatant was adjusted to 70% saturation with $(NH_4)_2SO_4$. The solution was again centrifuged at 10,000 x g for 20 minutes after stirring overnight and the precipitate obtained was then dissolved in 50 ml 0.2 M K_2HPO_4 and dialyzed against distilled water until free of sulfate. Any precipitate formed during dialysis was removed by centrifugation at 10,000 x g for 20 minutes. The supernatant (MT-C) was then lyophilized.

b) Molar Urine (MU).

24 hour molar urine having the total HCG immunological activity from 100,000 to 300,000IU was centrifuged at 5,000 x g for 10 minutes to remove precipitate. The clear supernatant was adjusted to pH 4 with 1 N HCl and stirred for 2 hours. The precipitate formed was removed by centrifugation at 10,000 x g for 20 minutes. The supernatant was then fractionated by the same salt precipitation procedures as described earlier for the molar tissue.

The above crude products (MT-C and MU-C) prepared from molar tissue and molar urine were subjected to further purification.

2. Fractionation by Immunoaffinity Chromatography:

a) Immunization and Preparation of Antiserum.

The immunizing method of Wong (1976) was followed. Each rabbit weekly received intramuscularly an immunizing dose of 1,000IU commercial U-HCG in 1 ml 1:1 (v/v) mixture of saline and Freund's complete adjuvant for the first 6 weeks. 7 days after the last injection, a test sample of blood was obtained from the ear vein of the rabbit. If the serum had reached an antibody titre of 1:10,000 using the aggulination reaction with HCG sensitized latex from the Pregnosticon Planotest Kit, booster injections were given every third week. About 20 ml blood was bled one week after each booster dose. Only sera with an antibody titre over 1:10,000 were useful. The crude antiserum was absorbed with lyophilized urinary proteins of non-pregnant women to remove extraneous antibodies. The absorbed antiserum was further concentrated by salt precipitation with $(NH_4)_2SO_4$. This treated antiserum which developed no precipitin lines to urinary proteins and human serum albumin on immunoelectrophoresis and immunodiffusion

was used to prepare the immunoadsorbant.

b) Preparation of Immunoadsorbant.

The HCG adsorbant was prepared by activation of Sepharose 6B with cyanogen bromide according to the method of Cuatrecasas (1970). 10 gm hydrated Sepharose 6B were activated with 1.8 gm finely ground cyanogen bromide for 15 minutes at pH 11. The activated gel suspension was thoroughly washed with cold PBS. 10 ml of treated antiserum was added to the washed gel. The mixture was gently agitated at $4^{\circ}C$ overnight. The coupled gel was washed extensively with PBS until no further protein was eluted and then successively with 2 M $MgCl_2$ at pH 6, 2 M NaTCA at pH 7, and again PBS. This anti-HCG IgG coupled Sepharose was stored at $4^{\circ}C$ in PBS until use.

c) Purification of Crude HCG Preparation by Chromatography by Anti-HCG IgG Sepharose.

The adsorption and desorption procedures adopted from Gospodarowicz (1972), Wong (1976), and Sairam et al. (1974) were employed to purify the crude HCG preparations (MT-C and MU-C). 2,000IU HCG dissolved in 5 ml PBS were charged onto an anti-HCG IgG Sepharose column (0.8 x 12 cm) previously equilibrated with PBS at room temperature. The column was then extensively washed with

PBS to remove all the non-specifically adsorbed proteins. And stepwise elution was performed successively with 2 M $MgCl_2$ (pH 6), 1 M NaTCA (pH 7), and 2 M NaTCA (pH 7). 2 ml fractions were collected at 4°C by a LKB-Utrorac 7000 fraction collector at a flow rate of 20 ml/hour. The elution was monitored by recording the absorbance of the eluates at 280nm by a LKB 8300 UV-cord II. Fractions obtained from each eluates were promptly pooled and dialyzed following elution. The PBS and $MgCl_2$ fractions were dialyzed against distilled water. The 1 M NaTCA and 2M NaTCA fractions which were previously diluted with distilled water, were dialyzed against several changes of 0.1 M NH_4HCO_3 for 2 days. After washing and equilibration with PBS, the affinity column was ready for another chromatography run. For mass production, only the 2 M NaTCA fractions were collected.

3. Fractionation by Chromatography on DEAE-Sephadex A-50.

All the steps described below were carried out at 4°C. The 2 M TCA fractions (MT-2MTCA and MU-2MTCA) were further purified batchwisely by chromatography on DEAE-Sephadex A-50. About 40,000IU sample dissolved in 5 ml 0.04 M Tris-phosphate buffer, pH 8.6, was dialyzed against the same buffer for 24 hours. Any precipitate formed during dialysis was removed by centrifugation. The clear

supernatant was applied to a DEAE-Sephadex A-50 column (1 x 30 cm) previously equilibrated with the above buffer. The column was initially eluted with 20 ml of the same buffer and then with 400 ml of a linear gradient from 0 to 0.2 m NaCl in 0.04 M Tris-phosphate buffer at pH 8.6. 5 ml fractions were collected at a flow rate of 30-50 ml/ hour. The elution was monitored by recording the absorbance of the eluate at 280nm. Appropriate fractions were pooled and dialyzed against 0.01 M NH_4HCO_3. Before lyophilization, the solution was centrifuged to remove the precipitate formed during dialysis.

A scheme for the purification of HCG from molar tissue and molar urine is shown in Fig. 1.

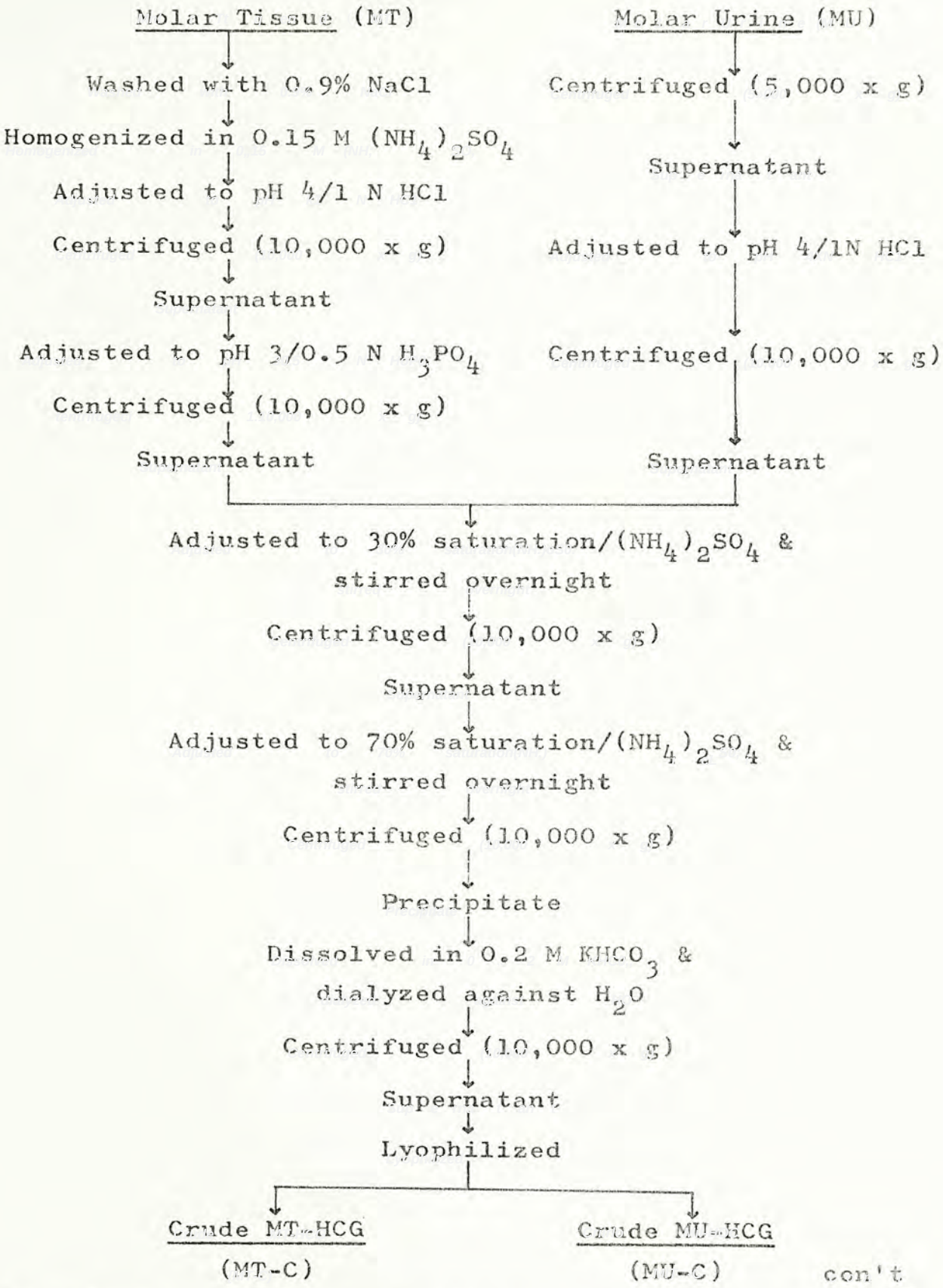
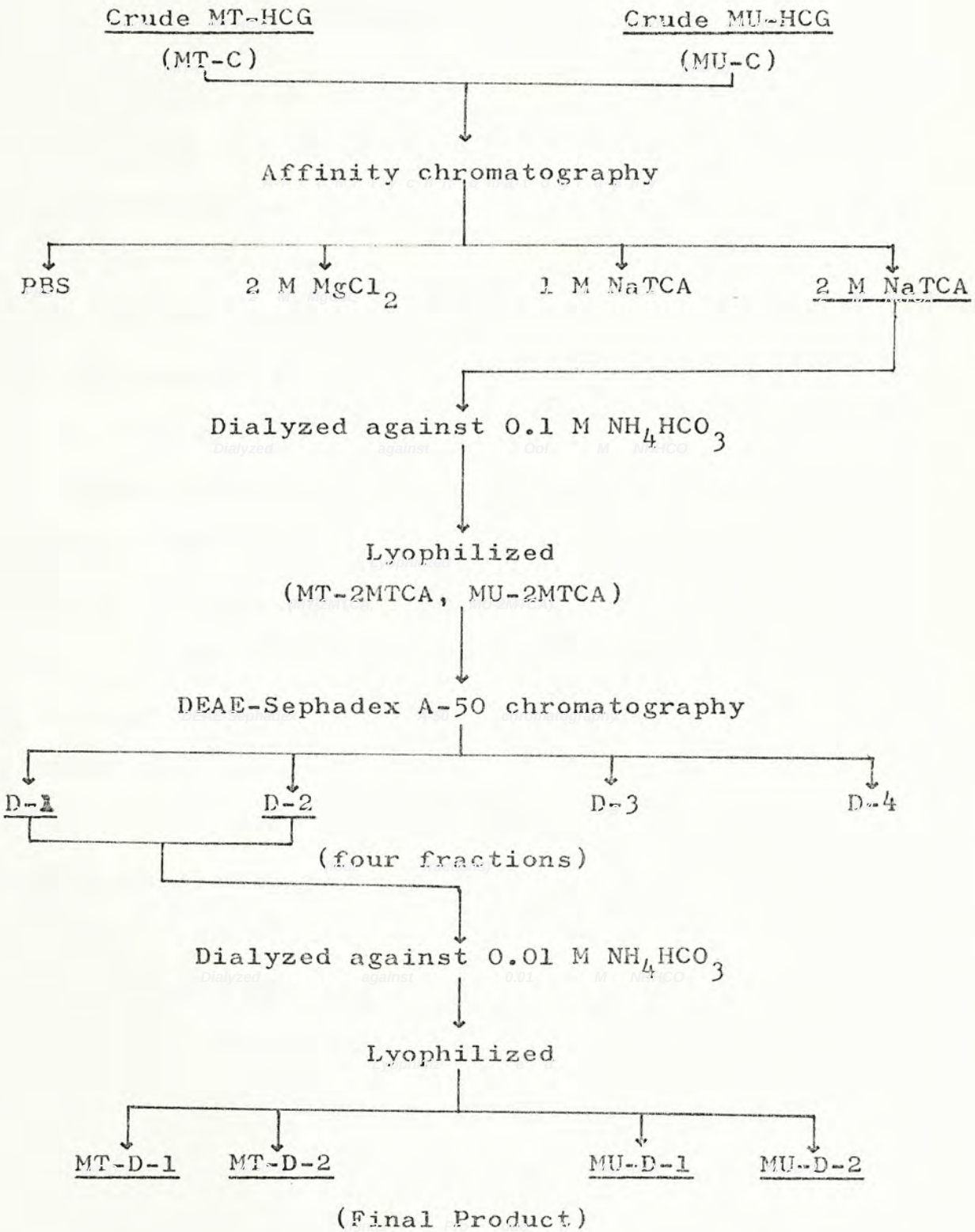


Fig. 1 Scheme for the purification of HCG from molar tissue and molar urine.

con't Fig. 1


Crude MT-HCG
(MT-C)
Crude MU-HCG
(MU-C)
Affinity chromatography
PBS
2 M $MgCl_2$
1 M NaTCA
2 M NaTCA
Dialyzed against 0.1 M NH_4HCO_3
Lyophilized
(MT-2MTCA, MU-2MTCA)
DEAE-Sephadex A-50 chromatography
D-1
D-2
D-3
D-4
(four fractions)
Dialyzed against 0.01 M NH_4HCO_3
Lyophilized
MT-D-1
MT-D-2
MU-D-1
MU-D-2
(Final Product)

PART II. CHARACTERIZATION STUDIES

1. Analytical Polyacrylamide Gel Electrophoresis.

Analytical disc electrophoresis was conducted on the purified HCGs and on all other fractions obtained during purification as described above. The disc technique of Davis (1964) was used with slight modifications. The spacer gel was composed of 3% (w/v) acrylamide at pH 6.7. The running gel of 7% (w/v) of acrylamide had a pH of 8.9. Tris-glycine, pH 8.3 was used as reservoir buffer. Sample with a concentration of 1 mg protein/ml in 30% sucrose solution was applied to each column. Electrophoresis was carried out at room temperature at a constant current of 3 mA per tube until the tracing dye (bromophenol blue) had reached the end of the column. The gels were stained with 0.25% Coomassie brilliant blue R250 for 3 hours. They were then destained in a solution made of methanol-glacial acetic acid-water (1:3:10).

In order to determine the activity distribution and its relation with the protein bands, after electrophoresis, the gel was cut longitudinally into two halves. One half was stained to reveal the protein bands and the other was cut into sections with 5 mm long. Each section was ground and extracted with PBS at $4^{\circ}C$ overnight. The gel was removed with centrifugation and the supernatant was assayed for its immunological activity.

2. N-terminal Amino Acid Determination.

To determine the N-terminal amino acids, all the free amino groups of the protein were dansylated according the procedure of Gray (1967). 0.5 mg sample dissolved in 0.5 ml of 0.5 M $NaHCO_3$ containing 8 M urea was coupled with 0.5 ml dansyl chloride (1-dimethylamino-naphthalene-5-sulphonyl chloride) in acetone (20 mg/ml) at room temperature overnight. The sample was dried under reduced pressure. The dry residue was dissolved in a small amount of distilled water and dialyzed against distilled was at $4^{\circ}C$ with several changes of water. After lyophilization, the sample was hydrolyzed with 50 μl of constant boiling HCl in a sealed and evacuated tube at $110^{\circ}C$ for 16 hours. The tube was then opened and dried in a desiccator containing NaOH pellets. The dansylated amino acids were separated and identified by polyamide layer chromatography described by Woods and Wang (1967). The dried hydrolyzate which was dissolved in a mixture of acetone and glacial acetic acid with a ratio of 3:2 (v/v) was spotted to a polyamide sheet. The following three solvent systems were used respectively for a three dimensional development.

a) 90% formic acid:water (1.5:100, v/v)

b) benzene:glacial acetic acid (9:1, v/v)

c) ethylacetate:methanol:glacial acetic acid (20:1:1, v/v)

After the first development, appropriate standard

dansyl amino acids were applied to the two sides of the polyamide sheet. The dansylated end terminal amino acids were visualized by ultraviolet irradiation.

3. Double Immunodiffusion.

The radial double diffusion method of Ouchterlony (1949) was followed. 1% agar in PBS was used for setting the agar in a petri dish. The wells were molded by short glass cylinders of 7 mm outside diameter with the peripheral wells at a distance of 2 cm from the central well. The central well was charged with enriched and absorbed antiserum. The peripheral wells were filled with antigen solutions. The petri dish was placed at room temperature for the immunodiffusion patterns to develop, which was completed for 7 days.

4. Immunoelectrophoresis.

The method described by Hamashige and Arquilla (1963) was performed with some modifications. To prepare the agarose slides, 0.8% agarose in pH 8.6 sodium barbital buffer of ionic strength 0.025 was coated to the degreased microscope slides. After the agarose solution had been gelled, two 0.5 mm wells were punched out of the gel about one third of the distance across the length of the slide. 5 μl antigen solution with a concentration of 5 mg/ml was

charged to the well. Sodium barbital solution, pH 8.6, with an ionic strength of 0.05 was used as electrophoretic tank buffer. Electrophoresis was performed at a constant voltage of 200 volts for 2 hours. After electrophoresis, a 2 mm trough was cut in the middle of the gel and filled with enriched and absorbed antiserum. The slide was then kept in a humid atmosphere for 30 hours for the development of precipitin lines by double diffusion.

For washing and drying, the method of Williams (1971) was followed. The developed slides were washed for 2 days with several changes of 2% NaCl solution made up in 0.05 M phosphate buffer at pH 8.0. The salt solution was then replaced with water for one day. To dry the slides, all wells and troughs were filled with water. The slide was then covered with a soaking wet sheet of filter paper and dried by a hair drier. Staining and destaining procedures were the same as those for the polyacrylamide gels which had been described earlier.

5. Molecular Weight Determination.

The sodium dodecyl sulfate (SDS) method described by Dunker and Rueckert (1969) was adopted for molecular weight determination. Polyacrylamide gel columns were prepared with 10% acrylamide in 0.05 M sodium phosphate buffer at pH 7 containing 1% SDS. Cytochrome C, chymotrypsinogen, bovine serum albumin, myoglobin and ovalbumin were used

as calibration markers. Before electrophoresis, the samples (2 mg/ml) and the markers (1 mg/ml) were dissolved in 0.01 M sodium phosphate buffer, pH 7.0, containing 1% (v/v) 2-mercaptoethanol, 1% (w/v) SDS and 4 M urea, and then incubated at 37°C for two hour. All the markers proteins were mixed and applied to one column while each HCG sample was mixed with cyctochrome C only and applied to each of other columns. Electrophoresis was carried out at a constant current of 7 mA per tube for 5 hours. The staining and destaining methods for the gels were the same as described earlier.

6. Optical Rotatory Dispersion (ORD) Studies.

ORD measurements of HCG preparations between 500 and 220nm were performed in 0.05 M NH_4HCO_3 buffer at 20°C using a Jasco Model ORD/UV-5 automatic recording spectropolarimeter. Solutions of 0.5 mg/ml and cuvettes of a path length of 1 cm were used. The equation of Moffitt (1956) was used to calculate the helical content in the protein.

7. Absorption Spectrum and Extinction Coefficient.

The absorption spectra of HCG preparations between 340 and 230nm were peformed in 0.05 M NH_4HCO_3 at room temperature (22°C) using a Beckman Model 25 spectrophometer. Solutions of 0.5 mg/ml and cuvettes of a path

length of 1 cm were used. The extinction coefficients, $E^{1\%}_{cm}$, at 280nm were calculated.

8. Amino Acid Composition Determination

Samples of 0.6 to 0.8 mg HCG were hydrolyzed with 1 ml of constant boiling HCl in an evacuated sealed tubes at 110°C for 22 hours. They were dried over NaOH and under vacuum in a desiccator. The resulting residue was dissolved in sodium citrate buffer (0.2 M Na^+), pH 2.2 Suitable aliquot was applied to each column of a Beckman Model 120C amino acid analyzer. Analysis was performed by the method of Spackman et al. (1958). Dot counting method was used to integrate the peak area on the chromatogram.

9. Carbohydrate Composition Determination

a) Neutral Sugars and Amino Sugars Determination.

The neutral sugars and amino sugars of HCG were determined qualitatively and quantitatively by gas liquid chromatography as their alditol acetate derivatives (Niedermeier, 1971; Niedermeier and Tomana, 1974). HCG samples were dried to constant weight in an evacuated desiccator. For neutral sugars determination, about 1 mg of HCG sample was hydrolyzed with 1 ml 1 N HCl in a sealed and evacuated tube at 100°C for 2 hours

(Van Hell, 1968; Niedermeier, 1971). For amino sugars, about 1 mg of HCG sample was hydrolyzed in 1 ml 2 N HCl in a sealed and evacuated tube at 105°C for 5 hours (Bahl, 1969a, Ashitaka, 1970). After cooling, the tube was opened and 0.2 ml internal standard solution containing 0.25 mole standard was added. For amino sugars, the internal standard was mannosamine and for neutral sugars, it was arabinose. The hydrolyzate was then neutralized to pH 6 with Dowex 1 HCO_3^-. The resin was removed by filtration. The free sugars in the filtrate was then reduced by an excess amount of sodium borohydride. Following reduction for 12 hours at 4°C, excess sodium borohydride was decomposed by the addition of 200 µl 6 N HCl and the sample was evaporated to dryness by the use of a rotatory evaporator. Borate was removed as volatile trimethyl borate by the addition of five 5 ml portions of methanol with concentration to dryness by a rotatory evaporator after each addition. For acetylation, 1 ml of pyridine and 1 ml of acetic anhydride was added to the dry residue and the flask was then capped. The sample was heated at 100°C for 20 minutes. After cooling, 3 ml distilled water was added. The pyridine together with water was co-distilled to dryness by a rotatory evaporator. This treatment was repeated 5 times. The dry acetylated mixture was dissolved in 20 µl chloroform. 1 µl of this aliquot

was injected into a Hewlett-Packard Model 402B gas chromatography, equipped with a dual-flame ionization detector with a single channel electrometer and 6 U-shape ¼" diameter glass columns. To determine the amino sugars, the column packed with 3% Poly A-103 on 100/120 mesh Gas Chrom. Q was used, and the chromatographic temperature was 230°C. For neutral sugars determination, the column used was packed with 3% ECNSS-M on 100/120 mesh Gas Chrom. Q and the temperature was 200°C. Standard alditol acetate derivatives for neutral sugars (containing equal molar amount of fucose, arabinose, mannose and galactose) and for amino sugars (containing mannosamine, galactosamine and glucosamine) were used to determined the retention times and the molar responses of the detector to the sugars relative to its responses to the internal standards.

b) Sialic Acid Determination.

Removal of sialic acid from samples were performed according to Codington et al. (1976). About 0.5 mg of HCG was hydrolyzed with 0.20 ml of 0.05 N H_2SO_4 at 80°C for 1 hour.

The liberated sialic acid was assayed by thiobarbituric method of Warren (1957). A calibration curve containing 4 to 20 μg of sialic acid was made.

RESULTS

PART I. PURIFICATION OF HCG FROM TROPHOBLASTIC TISSUE AND URINE OF PATIENTS WITH HYDATIDIFORM MOLE

1. Fractionation by Acid and Salt Precipitation.

Three batches of each sample (molar tissue and molar urine) were separately studied. The total activity in 70 gm molar tissue was found to range from 95,000 to 130,000IU; while 100,000 to 300,000IU were obtained in 24 hour molar urine. After acid and salt precipitation, both crude preparations had a recovery of 65 to 75% of total HCG activity. The white lyophilized crude products of molar tissue possessed specific activity in the range of 350 to 430IU/mg, which was higher than that of 115-190 IU/mg in the yellowish product from molar urine.

2. Fractionation by Immunoaffinity Chromatography.

For further purification of the crude preparations, about 2,000IU of HCG were charged onto an anti-HCG IgG column at each time. Proteins not bound to the column were eluted out as a large peak with PBS as shown in Fig. 2. Small protein peaks were obtained when stepwise elution was successively performed with 2 M $MgCl_2$, 1 M NaTCA, and 2 M NaTCA. HCG activity was found to be present only in

TABLE 5.1.15 AGE GROUP VS SPONSORSE SPONSORING PLAN OR NOT

DISTRIBUTORS' AGE GROUP		BASE	SPONSORS+SPONSORING PLAN NON SPONSO	YES	NO	NO ANSWER
		59	31	10	17	1
		100.0	100.0	100.0	100.0	100.0
		100.0	52.5	16.9	28.8	1.7
UNDER 20		0	0	0	0	0
	COL. %	0.0	0.0	0.0	0.0	0.0
	ROW %	100.0	0.0	0.0	0.0	0.0
20 - UNDER 30		38	20	8	9	1
	COL. %	64.4	64.5	80.0	52.9	100.0
	ROW %	100.0	52.6	21.1	23.7	2.6
30 - UNDER 40		13	5	1	7	0
	COL. %	22.0	16.1	10.0	41.2	0.0
	ROW %	100.0	38.5	7.7	53.8	0.0
40 - UNDER 50		6	4	1	1	0
	COL. %	10.2	12.9	10.0	5.9	0.0
	ROW %	100.0	66.7	16.7	16.7	0.0
OVER 50		2	2	0	0	0
	COL. %	3.4	6.5	0.0	0.0	0.0
	ROW %	100.0	100.0	0.0	0.0	0.0
NO ANSWER		0	0	0	0	0
	COL. %	0.0	0.0	0.0	0.0	0.0
	ROW %	100.0	0.0	0.0	0.0	0.0

the 2 M NaTCA fraction. The specific activity and recovery of typical batches of samples are shown in Table 1. The specific activities of HCG after affinity chromatography increased 6.5 and 11 folds respectively for the molar tissue and molar urine preparations. Both samples had a recovery of about 70%.

3. Fractionation by Chromatography on DEAE-Sephadex A-50

About 30,000 to 40,000IU MT-HCG and MU-HCG collected from the above 2 M NaTCA fractions were respectively subjected DEAE-Sephadex chromatography and eluted with a linear gradient between 0 to 0.2 M NaCl in Tris-phosphate buffer. The elution patterns are shown in Fig. 3. The two patterns are quite similar both in HCG activity and protein distribution in the first half of elution. However, for MU-2MTCA, large amount of protein with very low HCG activity was eluted out at the second half of elution. The eluate was separated into 4 fractions designated as D-1, D-2, D-3 and D-4. The activity of each fraction was determined and the results were shown in Table 2. It was found that most of the HCG was desorbed from the ion-exchanger before the concentration of the eluate had reached 0.12 M NaCl. D-1 and D-2 were the most active fractions. Though D-1 fractions in both preparations possessed the higher specific activity, most of the activity was found in the D-2 fractions.

Table 1. Specific Activity and Recovery after Immunoaffinity Chromatography.

Product / Sample	Crude		2 M NaTCA Fraction	
	Activity IU	Specific Activity IU/mg	% Activity Recovered	Specific Activity IU/mg
Molar Tissue	2,000	435	*70.0 +(66.0-75.6)	*2,830 (2,300-3,400)
Molar Urine	2,000	115	**69.6 (61.2-73.5)	**1,260 (1,070-1,360)

*Mean values from 21 experiments with the same batch of sample.

**Mean values from 26 experiments with the same batch of sample.

+Figures in the parentheses indicate the range.

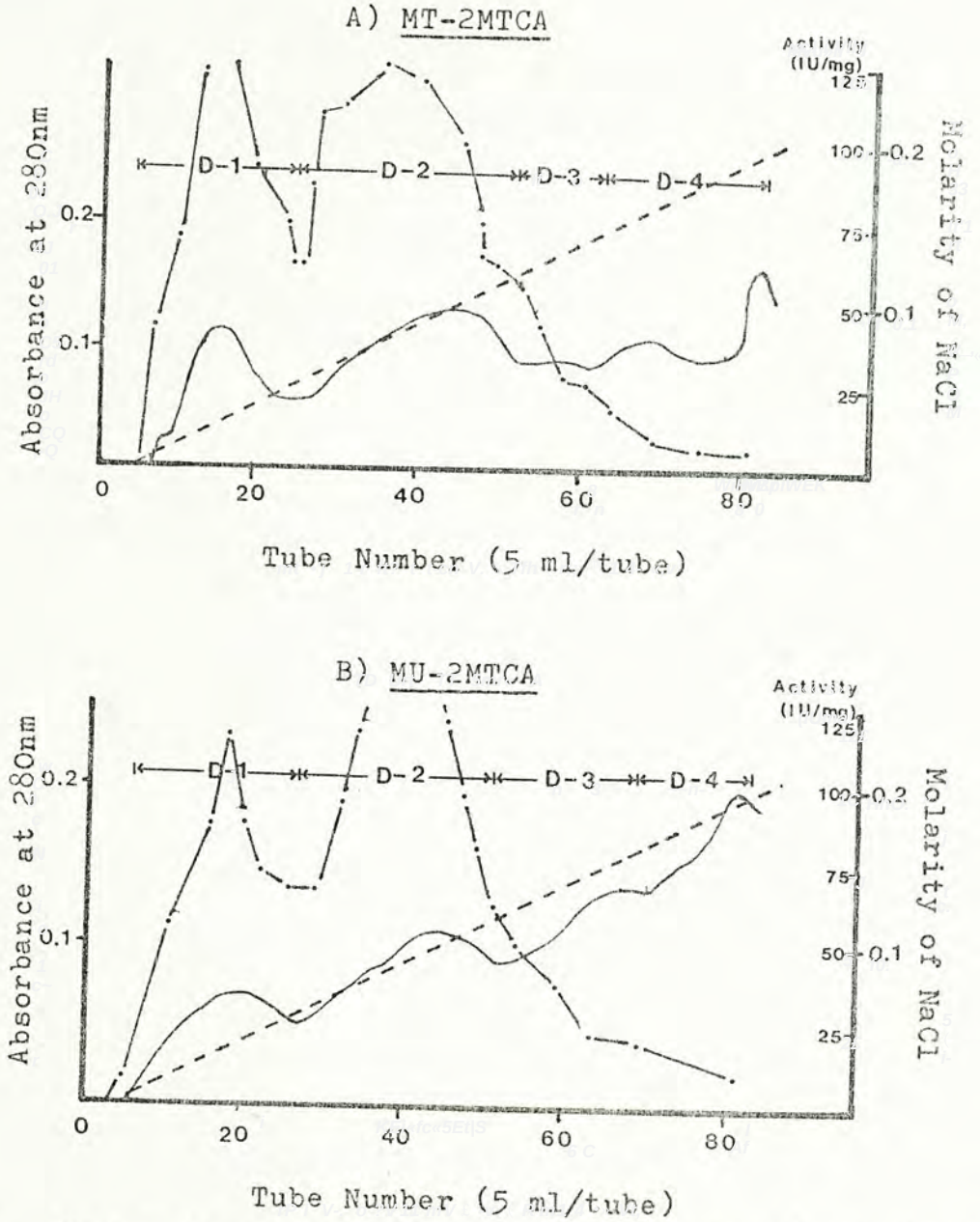


Fig. 3. Chromatography of A) MT-2MTCA and B) MU-2MTCA fractions on DEAE-Sephadex A-50 columns (1 x 30 cm) equilibrated with 0.04 M Tris-phosphate buffer. The column were eluted with linear gradient between 0 to 0.2 M NaCl in the above buffer.

⟵ ⟶ Fractions pooled
———— Optical density at 280nm
------- Molarity of NaCl
—·—·—·— HCG immunological activity

Table 2. Activity and Protein Content Recovered after DEAE-Sephadex Chromatography.

A. Molar Tissue

Fraction	Total Activity IU	Protein Content mg	Specific Activity IU/mg
MT-2MTCA	30,000	10.60	2,830
MT-D-1	4,900	0.62	7,900
MT-D-2	16,200	2.60	6,230
MT-D-3	1,590	1.45	1,100
MT-D-4	454	2.30	216

B. Molar Urine

Fraction	Total Activity IU	Protein Content mg	Specific Activity IU/mg
MU-2MTCA	40,000	31.74	1,260
MU-D-1	3,150	0.60	5,250
MU-D-2	18,680	4.57	4,090
MU-D-3	3,540	6.62	540
MU-D-4	1,050	7.91	130

The results of typical purification schedules for the HCGs extracted from molar tissue and molar urine are shown in Tables 3A and 3B respectively.

Table 3A. Recovery and Specific Activity of HCG from Molar Tissue during Purification.

Fractionation Procedure	Fraction	Total Activity (IU)	Specific Activity (IU/mg)	% Recovery
	70gm Molar Tissue	110,000	180	100.0
Salt Precipitation	MT-C	78,000	435	71.0
Affinity Chromatography	MT-2MTCA	54,600	2,830	49.7
DEAE-Sephadex Chromatography	MT-D-1	8,920	7,900	8.1
	MT-D-2	29,500	6,230	26.8

*** THE CHINESE UNIVERSITY OF HONG KONG ***

LINGNAN INSTITUTE OF BUSINESS ADMINISTRATION

MASTER THESIS - DIRECT SELLING SURVEY BY HO YU-CHOI

TABLE 5.1.16 AGE VS NUMBER OF NEW DISTRIBUTORS TO BE SPONSORED BY THE SPONSOR

DISTRIBUTORS' AGE GROUP		NUMBER OF NEW DISTRIBUTORS TO BE SPONSORED BY THE SPON									
		NON	ONE	3	5	7	9	OVER 9	DON'T KNOW	ZERO	
	BASESPONSODISTRI	59	31	8	4	2	0	0	1	11	2
		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
		100.0	52.5	13.6	6.8	3.4	0.0	0.0	1.7	18.6	3.4
UNDER 20		0	0	0	0	0	0	0	0	0	0
	COL. %	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	ROW %	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
20 - UNDER 30		38	20	5	4	1	0	0	1	6	1
	COL. %	64.4	64.5	62.5	100.0	50.0	0.0	0.0	100.0	54.5	50.0
	ROW %	100.0	52.6	13.2	10.5	2.6	0.0	0.0	2.6	15.8	2.6
30 - UNDER 40		13	5	1	0	1	0	0	0	5	1
	COL. %	22.0	16.1	12.5	0.0	50.0	0.0	0.0	0.0	45.5	50.0
	ROW %	100.0	38.5	7.7	0.0	7.7	0.0	0.0	0.0	38.5	7.7
40 - UNDER 50		6	4	2	0	0	0	0	0	0	0
	COL. %	10.2	12.9	25.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	ROW %	100.0	66.7	33.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
OVER 50		2	2	0	0	0	0	0	0	0	0
	COL. %	3.4	6.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	ROW %	100.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
NO ANSWER		0	0	0	0	0	0	0	0	0	0
	COL. %	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	ROW %	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

PART II. CHARACTERIZATION STUDIES

Although two active fractions (D-1 and D-2) were purified from both molar tissue and molar urine, the small amount of the D-1 fraction could not afford all the characterization studies described in 'Materials and Methods'. Listed in the following are only the results on the D-2 fractions unless specified.

1. Analytical Polyacrylamide Gel Electrophoresis.

The electrophoretic patterns of all the fractions in 7% polyacrylamide gel at pH 8.9 are shown in Fig. 4A (molar tissue) and 4B (molar urine). More than 4 protein bands can be observed in the MT-2MTCA and MU-2MTCA fractions. However, the MU-D-1, MU-D-2, MT-D-1 and MT-D-2 fractions revealed only one but diffused band. The D-1 fractions were found to have lower mobilities than those of the D-2 fractions in the electrophoretograms.

Fig. 5 shows the electrophoretic patterns of MT-HCG (D-2), MU-HCG (D-2) and commercial U-HCG. Fig. 6 illustrates the electrophoretic protein patterns with HCG activity distribution of these three HCGs. The protein bands of MU-HCG and MT-HCG had similar HCG activity distribution. While protein bands with no HCG activity were found in commercial U-HCG.

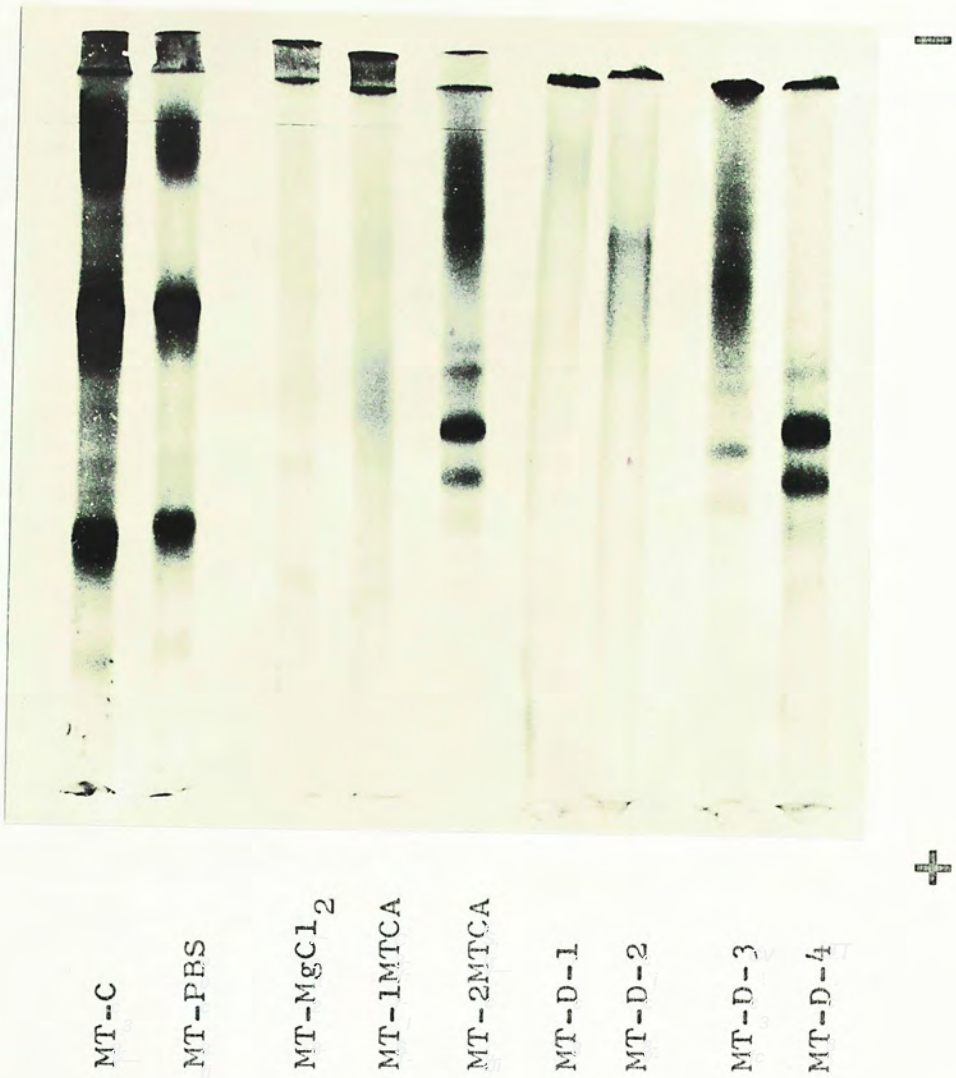


Fig. 4A. Disc electrophoresis of all the molar tissue fractions in 7% polyacrylamide gel at pH 8.9.

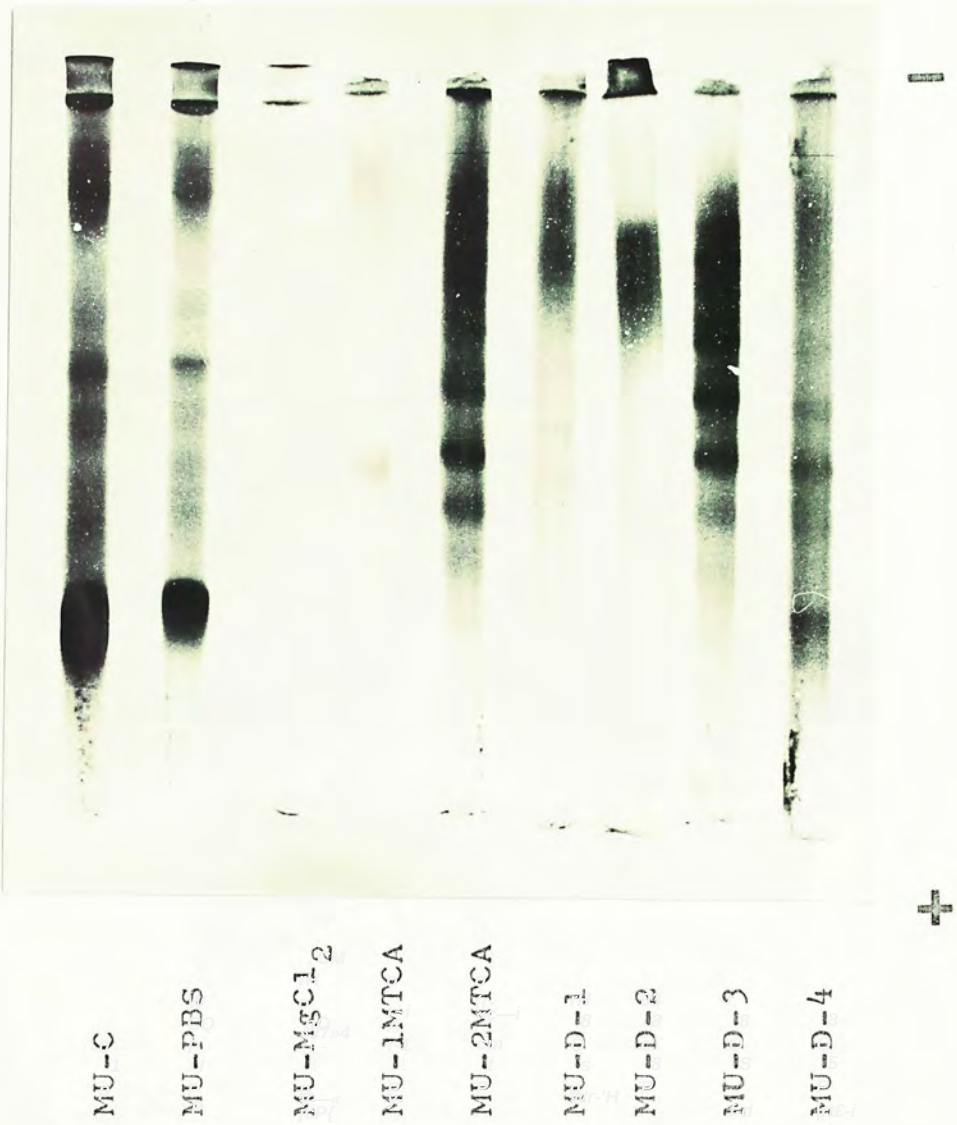


Fig. 4B. Disc electrophoresis of all the molar urine fractions in 7% polyacrylamide gel at pH 8.9.

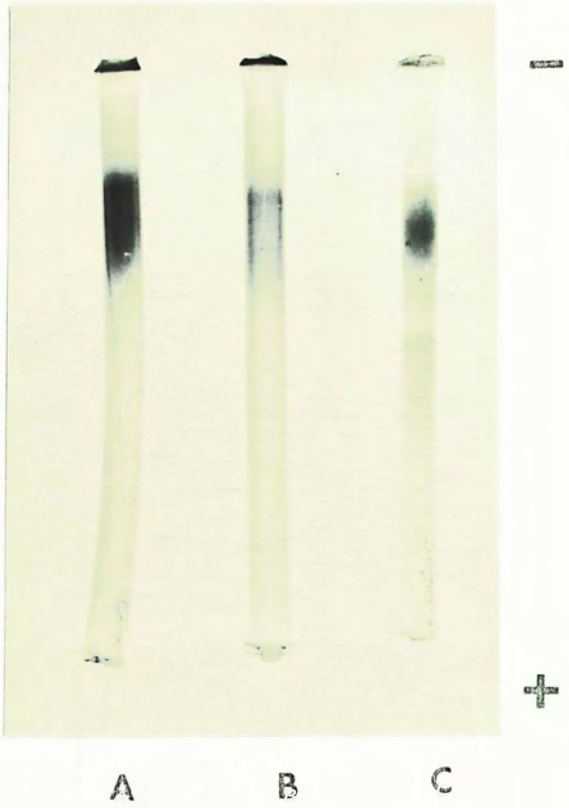


Fig. 5. Disc electrophoretic pattern of A) MU-HCG, B) MT-HCG and C) commercial U-HCG in 7% polyacrylamide gel at pH 8.9.

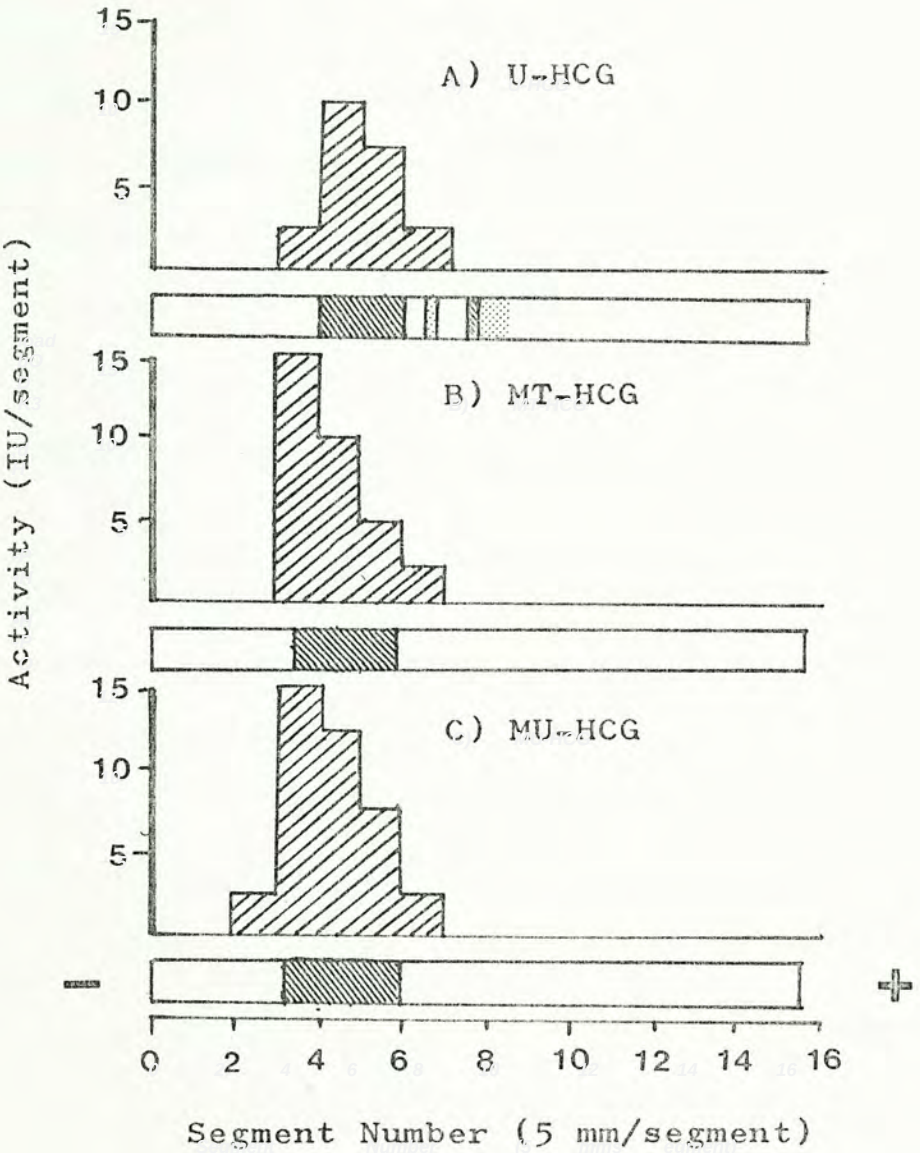


Fig. 6. Electrophoretic protein patters with HCG activity distribution of A) commercial U-HCG, B) MT-HCG and C) MU-HCG.

2. N-terminal Amino Acid Determination.

Figs. 7A and 7B are the 3 dimensional chromatograms of the hydrolysates of dansylated MT-HCG and MU-HCG on polyamide sheets. The relative intensities of the dansylated amino acids under ultraviolet radiation are also shown. The dansylated amino acids in the chromatograms were identified by comparision with the standard chromatogram of Woods and Wang (1967) and the standards that added to the polyamide sheets after the first dimensional development. As can be seen in Fig. 7A, serine and alanine are the major N-terminal amino acids in MT-HCG. Besides, a small amount of valine together with traces of aspartic or glutamic acid were also observed. The chromatogram of MU-HCG was similar to that of MT-HCG but with additional isoleucine and glycine existed in trace amounts.

3. Double Immunodiffusion

Figures 8A to 8F show the double immunodiffusion patterns of various fractions obtained during purification. Two apparent precipitin lines were observed for commercial U-HCG. MT-D-2 and MU-D-2 developed only one precipitin line, they fused completely with each other and with the inner line of commercial U-HCG (Fig. 8B). This inner line of U-HCG is derived from the native HCG and its specific antibody. The outer line arised from U-HCG was found to

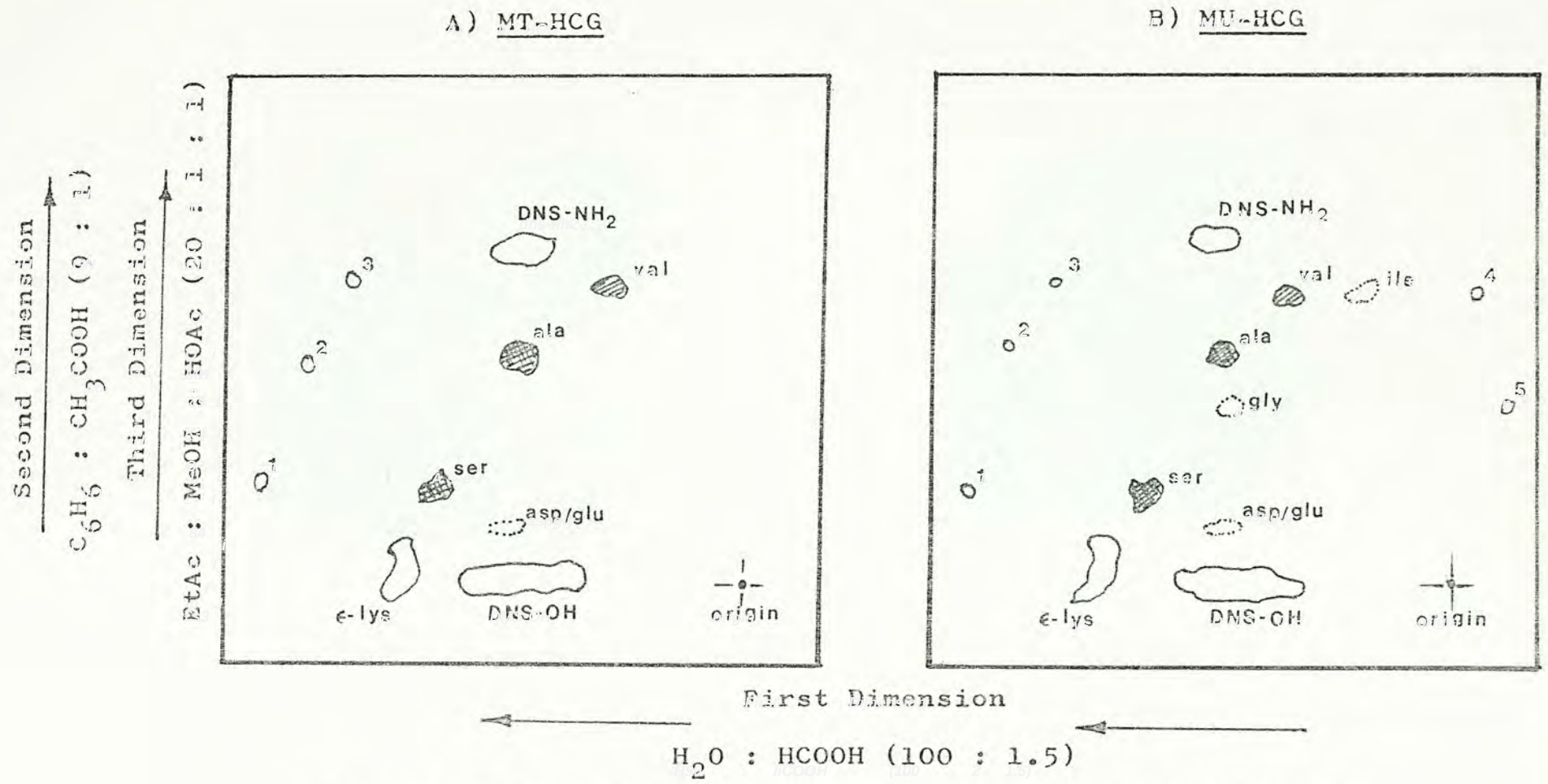


Fig. 7. Polyamide layer chromatography of the hydrolysates of dansylated A) MT-HCG and B) MU-HCG. Three dimensional developments were performed with various solvent systems as indicated. The dansyl amino acids applied as standards are: 1. serine; 2. alanine; 3. valine; 4. isoleucine; 5. glycine.

MT-2MTCA MU-2MTCA

U-HCG

MU-2MTCA MT-2MTCA

(Fig. 8A)

MT-D-2 MU-D-2

U-HCG

U-HCG

MU-D-2 MT-D-2

(Fig. 8B)

Figs. A-F. Double immunodiffusion patterns of various fractions obtained during purification. Anti-HCG antiserum was added into the central wells, while the various fractions were added to the peripheral wells as indicated.

*** THE CHINESE UNIVERSITY OF HONG KONG ***

LINGNAN INSTITUTE OF BUSINESS ADMINISTRATION

MASTER THESIS - DIRECT SELLING SURVEY BY HO YU-CHOI

TABLE 5.1.19 AGE GROUP VS LIMITATION OF THIS SYSTEM

DISTRIBUTORS' AGE GROUP		LIMITATION OF THE SYSTEM NON BASESPON.	NO	YES	DON+T KNOW	
		59	31	13	13	2
		100.0	100.0	100.0	100.0	100.0
		100.0	52.5	22.0	22.0	3.4
UNDER 20		0	0	0	0	0
	COL. %	0.0	0.0	0.0	0.0	0.0
	ROW %	100.0	0.0	0.0	0.0	0.0
20 - UNDER 30		38	20	8	9	1
	COL. %	64.4	64.5	61.5	69.2	50.0
	ROW %	100.0	52.6	21.1	23.7	2.6
30 - UNDER 40		13	5	4	4	0
	COL. %	22.0	16.1	30.8	30.8	0.0
	ROW %	100.0	38.5	30.8	30.8	0.0
40 - UNDER 50		6	4	1	0	1
	COL. %	10.2	12.9	7.7	0.0	50.0
	ROW %	100.0	66.7	16.7	0.0	16.7
OVER 50		2	2	0	0	0
	COL. %	3.4	6.5	0.0	0.0	0.0
	ROW %	100.0	100.0	0.0	0.0	0.0
NO ANSWER		0	0	0	0	0
	COL. %	0.0	0.0	0.0	0.0	0.0
	ROW %	100.0	0.0	0.0	0.0	0.0

to contain at least two components. None of the molar tissue fractions revealed the existence of these non-HCG components (Figs. 8A, C and D). On the contrary, small amounts of these antigens were found in MU-2MTCA (Figs. 8A and E). However, they were readily separated by DEAE-Sephadex chromatography and existed as major components in the MU-D-3 and MU-D-4 fractions (Fig. 8F). No precipitin lines were developed from MT-PBS, MT-$MgCl_2$, MT-1MTCA, MU-PBS and MU-$MgCl_2$. Diffused precipitin lines which cross-reacted with the HCG line were observed for MU-1MTCA and MT-D-4 (Figs. 8D and E). This observation probably suggests the existence of degraded HCG or antigens that have similar HCG-like determinants in these fractions.

4. Immunoelectrophoresis.

The microimmunoelectrophoretic patterns of HCG preparations are shown in Fig. 9. Most of the minor bands could be seen after staining. Several precipitin lines arised from commercial U-HCG and MU-2MTCA. But only one precipitin line was observed for MU-D-2, MT-2MTCA and MT-D-2.

5. Molecular Weight Determination.

The SDS electrophoretic patterns for MU-HCG, MT-HCG and commercial U-HCG are shown in Fig. 10. Cytochrome C was found to have two contaminated bands in addition to its

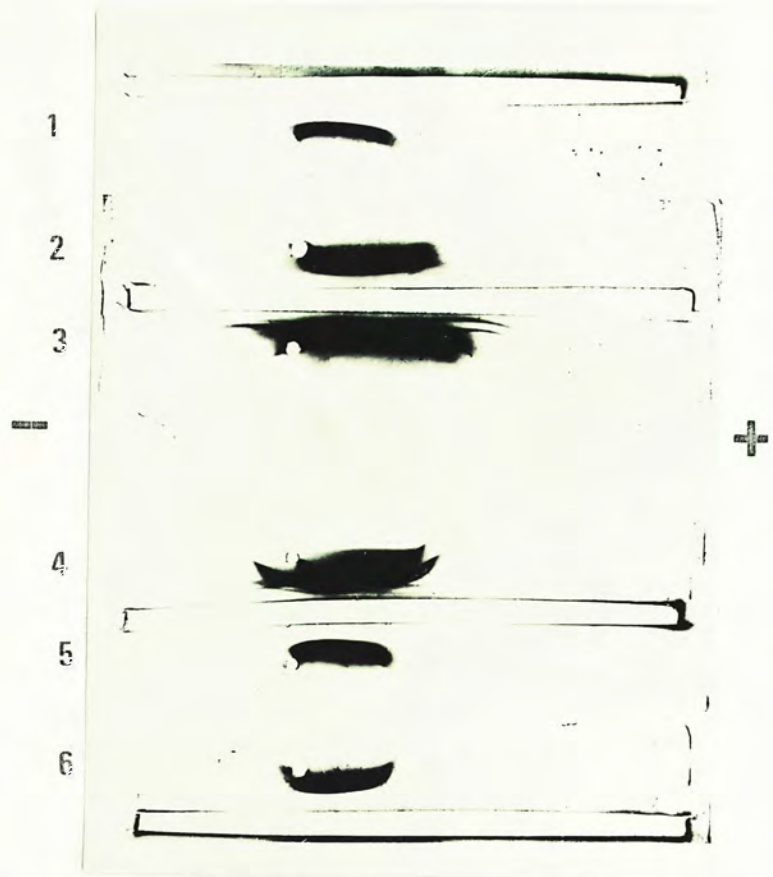


Fig. 9. Immunoelectrophoresis of HCG fractions. Samples applied to the wells were: 1, MU-D-2; 2, MU-2MTCA; 3 and 4, commercial U-HCG; 5, MT-2MTCA; 6, MT-D-2. After electrophoresis, anti-HCG antiserum was added to the troughs.

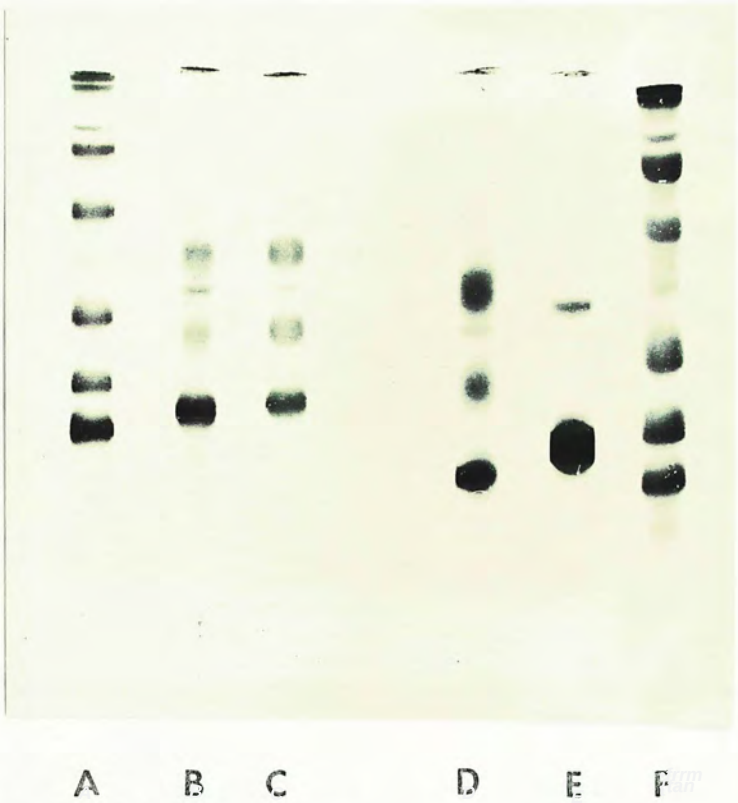


Fig. 10. SDS electrophoresis of HCG preparations. B, U-HCG; C, MT-HCG; D, MU-HCG; E, Cytochrome C. A and F, protein markers: the five intense bands from top to bottom are bovine serum albumin, ovalbumin, chymotrypsinogen A, myoglobin and cytochrome C. The lowest intense bands in B, C and D were derived from cytochrome C.

major band. Besides the cytochrome C bands, each HCG samples possessed two diffused intense bands. All the upper bands corresponded to a molecular weight of 31,000; while for the lower bands, it was 20,000. These two bands were undoubtedly identified to be the α and β subunits of the hormone. The molecular weight of the native HCGs was estimated to be 51,000 by the sum of those of the subunits.

6. Optical Rotatory Dispersion Studies.

The ORD spectra for both MT-HCG and MU-HCG between 220 and 500nm were practically identical (Fig. 11). When the Moffitt equation was employed to calculate the helical content of HCG, the result tended to be zero.

7. Absorption Spectrum and Extinction Coefficient.

The UV absorption spectra of MT-HCG and MU-HCG are shown respectively in Figs. 12A and 12B. Both spectra had absorption maxima at 278nm. Extinction coefficients at 280nm were calculated on a weight basis. The results are nearly the same as shown in Table 4.

8. Amino Acid Composition Determination.

The amino acid compositions of MU-HCG and MT-HCG

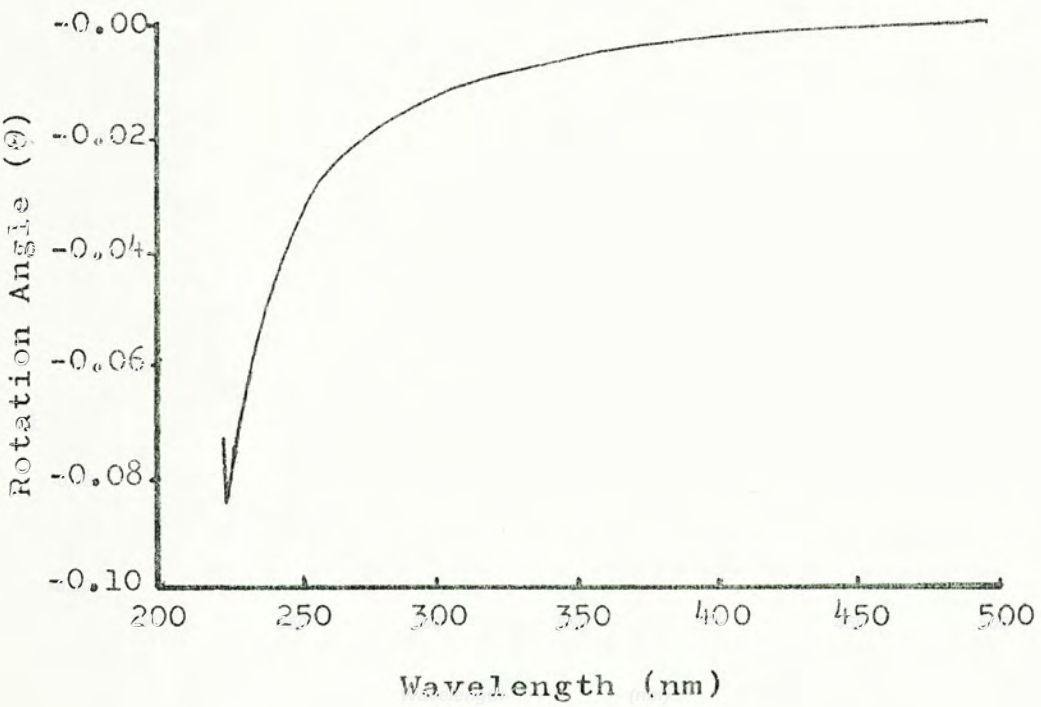


Fig. 11. Optical Rotatory Dispersion Spectra of MU-HCG and MT-HCG (they were practically identical and coincided with each other)

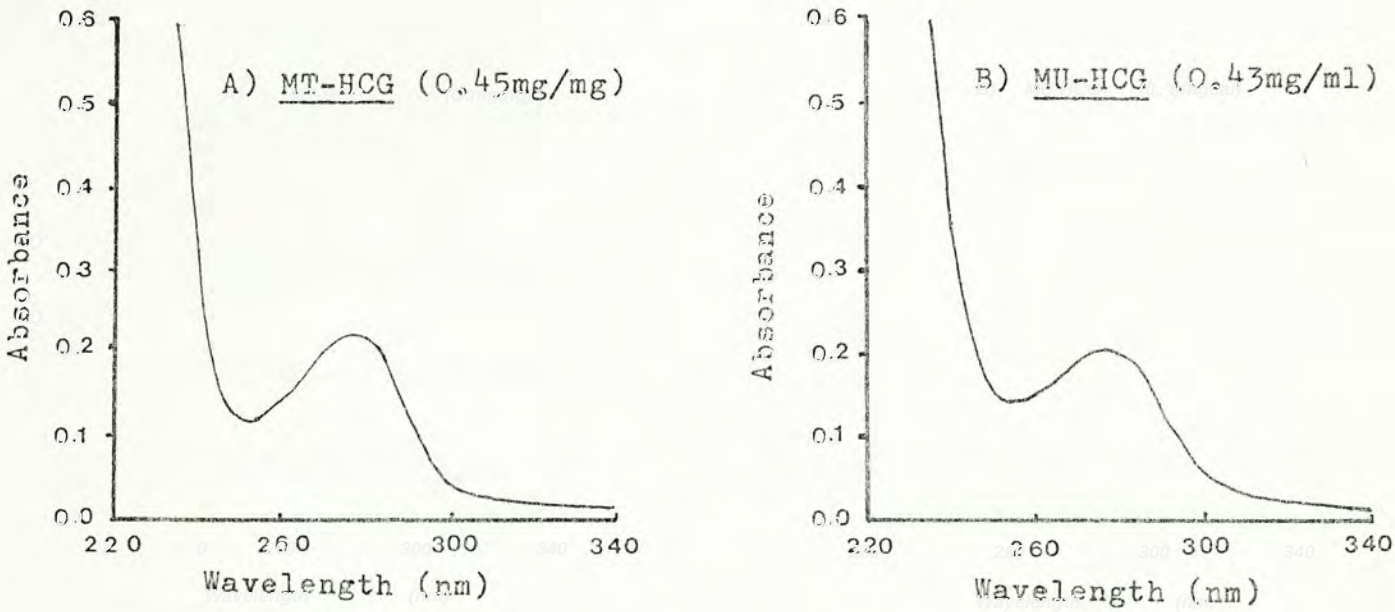


Fig. 12. UV spectra of A) MT-HCG and B) MU-HCG.

Table 4. Extinction coefficients of MT-HCG and MU-HCG at 280nm.

Sample	$E^{1\%}_{280nm}$ (cm^{-1})
MT-HCG	4.93
MU-HCG	4.86

were determined. The results based on 4 histidine residues per HCG molecule are shown in Table 5. No corrections were made for losses due to decomposition during hydrolysis. It can be seen that the two HCG preparations have very similar amino acid compositions.

9. Carbohydrate Composition Determination.

a) Neutral Sugars and Amino Sugars Determination.

Neutral sugars determination was done on column packed with 3% ECNSS-M on Gas Chrom Q. The detector responses and retention times of some neutral sugar derivatives relative to arabinose are listed in Table 6. The results on the neutral sugar compositions of MU-HCG and MT-HCG are presented in Table 7.

Column packed with 3% Poly A-103 on Gas Chrom. Q was employed for amino sugars determination. Table 8 listed the relative molar response factors and retention times of some amino sugar derivatives relative to mannosamine. The contents of each amino sugar present in the two HCG preparations are shown in Table 9.

b) Sialic Acid Determination.

Table 10 shows the sialic acid contents of the MU-D-1,

MU-D-2, MT-D-1 and MT-D-2 fractions. As can be seen in the same Table, the D-2 fractions have higher sialic content than the D-1 fractions.

Table 5. Amino Acid Compositions of MT-HCG and MT-HCG.[a]

Amino Acid	MT-HCG[c]	MU-HCG[b]
Lysine	9.9	10.0
Histidine	4.0	4.0
Arginine	10.6	10.6
Aspartic Acid	16.6	16.9
Threoine	16.7	16.8
Serine	16.5	16.8
Glutamic Acid	19.2	19.3
Proline	25.7	25.6
Glycine	12.6	13.3
Alanine	11.3	12.2
Half Cystine	12.8	11.1
Valine	16.7	17.0
Methionine	3.7	3.5
Isoleucine	6.4	6.3
Leucine	15.5	15.6
Tyrosine	6.0	6.3
Phenylalanine	7.3	7.6

[a] Results were calculated on the basis of 4 histidine residues.

[b] Mean values from three batches of sample.

[c] Mean values from two batches of sample.

Table 6. Detector Response Factors and Retention Times Relative to Arabinose for Alditol Acetates of Neutral Sugars.

Neutral Sugar	*Molar Response Factor	Retention Time
Fucose	0.82	0.72
Arabinose	1.00	1.00
Mannose	0.85	2.51
Galactose	0.82	2.84

* Average of 7 determinations.

Table 7. Neutral Sugar Contents of MT-HCG and MU-HCG.

Neutral Sugar	gm/ 100 gm dry sample	
	MT-HCG[a]	MU-HCG[b]
Fucose	+	+
Mannose	2.3	2.1
Galactose	5.5	6.7

[a] Mean values from 2 batches of sample.

[b] Mean values from 3 batches of sample.

+ Signifies present in small amount.

Table 8. Detector Response Factors and Retention Times Relative to Mannosamine for Alditol Acetates of Amino Sugars.

Amino Sugar	*Molar Response Factor	Retention Time
N-acetylgalactosamine	0.93	0.88
N-Acetylglucosamine	1.04	0.70
N-Acetylmannosamine	1.00	1.00

* Average of 5 determinations.

Table 9. Amino Sugar Contents of MT-HCG and MU-HCG.

Amino Sugar	gm/ 100 gm dry sample	
	MT-HCG*	MU-HCG*
N-acetylglucosamine	4.8	5.4
N-acetylgalactosamine	+	+

* Mean values from 2 batches of sample.

+ Signifies present in small amount.

Table 10. Sialic Acid Contents of HCG Fractions.

Fraction	gm sialic acid/ 100 gm dry sample
MT-D-1	2.8
MT-D-2[a]	6.2
MU-D-1	3.5
MU-D-2[b]	6.1

[a] Mean values from 2 batches of sample.

[b] Mean values from 3 batches of sample.

DISCUSSION

PART I. PURIFICATION OF HCG FROM TROPHOBLASTIC TISSUE AND URINE OF PATIENTS WITH HYDATIDIFORM MOLE

1. Fractionation by Acid and Salt Precipitation:

The total activities in both molar tissue and molar urine were found to differ from batch to batch. This is probably due to the differences in the degree of illness of the patients. The high recovery (65-75%) after acid and salt precipitation indicates that HCG is stable in acidic pH and high concentration of ammonium salt. It has also been revealed by Chan (1973) that most of the HCG can be precipitated between 30% and 70% saturation of ammonium sulfate.

2. Fractionation by Affinity Chromatography.

Before coupled to the sepharose, the crude antiserum was absorbed with urinary proteins of non-pregnant women in order to remove extraneous antibodies. The absorbed serum was then concentrated by precipitation with ammonium sulfate. This step not only increased the HCG capacity of the affinity column but also minimized the risk of non-specific binding of protein (Gospodarowicz, 1972). The capacity of our affinity column was about 2,000IU.

Because of the stable binding of HCG to its specific antibody, difficulty in the selection of a good desorbing eluate had once been the problem we encountered. Even 8 M urea was inefficient to elute the HCG molecules from the affinity column. 4 M $MgCl_2$, which has been reported to dissociate most of the antibody-antigen complexes (Avrameas and Ternynck, 1969), desorbed only a small amount of the HCG molecules. In the purification of HCG from placentas, Wong (1976) of this laboratory used successively 4 M $MgCl_2$ (pH 6) and 6 M guanidine HCl (pH. 1.5) as eluates. 20% and 50% of the total activity applied were recovered respectively in the $MgCl_2$ and guanidine HCl fractions. It was also found that when exposed to 6 M guanidine HCl (pH 1.5) HCG was dissociated into its subunits and dialysis for several days was necessary for reconstituting the hormone. However, when the same eluting system was employed for the purification of molar HCG, both the yield and the extent of purification were found to be low. Wilched and Gorecki (1973) found that guanidine HCl which was employed to remove counter-ligand from antibody sepharose conjugates caused destruction rather than regeneration of immunoaffinity columns. More recently, Sairam *et al.* (1974) suggested that the use of highly acidic solutions or denaturants (guanidine HCl or urea) or a combination of both in the dissociation of antigen-antibody complexes on column frequently would lead to undersirable damage of many proteins.

These observations probably indicate the risk in using guanidine HCl as desorbing agent.

In the present study, 2 M $MgCl_2$ (pH 6), 1 M NaTCA (pH 7) and 2 M NaTCA (pH 7) were successively used as eluates. It was found that part of the non-specific bound proteins were eluted by 2 M $MgCl_2$ and 1 M NaTCA, while the elution of HCG was accomplished by 2 M NaTCA. After affinity chromatography, the specific activities increased from 435 to 2830IU/mg for MT-HCG and from 115 to 1,260IU/mg for MU-HCG. The similar recovery, about 70%, in the 2 M NaTCA fractions for both molar tissue and molar urine preparations probably suggests that MT-HCG and MU-HCG are immunologically similar. As the affinity columns were found to be effective in repeated use without any decrease in their capacity, it seemed that the eluates we used did not give any damage to the immobilized anti-HCG IgG molecules.

Trifluoroacetate and trichloroacetate in neutral solutions were first introduced by Sairam et al. (1974) to dissociate antigen-antibody complexes in affinity chromatography. It has also been suggested by the same group of authors that these ions in neutral solutions are less drastic than the conventional deforming solvents of extreme pH or ionic strength or containing denaturants such as urea and guanidine HCl. The effective use of 2 M NaTCA as eluate in our present study seems to further support this suggestion.

3. Fractionation by Chromatography on DEAE-Sephadex A-50

Two active fractions, D-1 and D-2 were obtained from both molar tissue and molar urine after DEAE chromatography (Table 2). The D-1 fractions showed a higher specific activity than the D-2 fractions. However, the total HCG activity in both D-1 fraction was low, only about one-fifth of the D-2 fraction. In disc electrophoresis, D-1 exhibited one diffused band but with a lower mobility than D-2 (Figs. 3A and B). Immunodiffusion studies showed that all the D-1 and D-2 fractions were immunologically identical. In addition, the D-1 fractions were found to have lower sialic acid content (Table 10). In the studies of the immunological and physical properties of HCG secreted by hydatidiform mole and trophoblastic tumours, Vaitukaitis and his coworkers (Vaitukaitis, 1973; Vaitukaitis et al., 1976; Vaitukaitis and Ebersole, 1976) found that altered forms of HCG with slight difference in molecular weight were present in the clinical materials studied. In view of the characteristics of D-1 and D-2 discussed above, the physicochemical differences between them are probably due to the variations in carbohydrate structure (see also discussion in 'Immunological Studies' on p. 75). And the differences in their specific activities may be reasoned by their different intrinsic activities and/or degree of purity between them. However, the small yields of MU-D-1 and MT-D-1 prevented further characterization.

Ashitaka and his coworkers (Ashitaka, 1970; Ashitaka et al., 1972) reported that HCG preparations obtained from various sources were found to be composed of two components. One has the LH-like activity and the other has FSH-like activity. The relative distribution of these two components varies according to the sources studied. HCG derived from urine of molar patient has the most dominant FSH-like activity comprising 5% of the total biological activity. This FSH-like component which exists in only small amount has very low immuno-specific activity (less than 100IU/mg in U-HCG and a higher mobility than the major component (LH-like) when studied by polyacrylamide disc electrophoresis. As can be seen, these characteristics are obviously in contrast with those of our D-1 and D-2 fractions, so Ashitaka's findings are not the case for ours.

After DEAE chromatography, the specific activity of the major fractions (D-2) increased by 2.2 and 3.3 folds for the preparations obtained from molar tissue and molar urine respectively. This observation indicates that MU-2MTCA which is collected just after affinity chromatography probably has more non-HCG contaminants than MT-2MTCA (will be discussed in details on p. 75, 'Immunological Studies'). When the results on DEAE chromatography are viewed together with those of disc electrophoresis and immunological studies, this ion-exchange chromatographic step is necessary and effective in isolating the non-HCG components in MU-2MTCA

and MT-2MTCA.

The total recoveries of HCG activities from molar tissue and molar urine were 27% and 22% respectively after the above three fractionation steps. For MT-HCG, it possessed a specific activity of 6,230IU/mg, a 35-fold increase when compared with the starting material. While for MU-HCG, its specific activity was 4,090IU/mg, an increase of 45 folds. The different specific activities for the two HCG preparations may also be reasoned by their differences in intrinsic activity and/or degree of purity.

Chan (1973) of this laboratory, using salt precipitation and DEAE-Sephadex ion exchange chromatography in conjunction with Sephadex G-200 and Sephadex G-100 gel filtration, obtained highly purified HCG from molar tissue with specific activity of 21,000IU/mg, a 20-fold increase from the starting material. Its yield was 19.7%. As can be seen, our final products possess a lower specific activity than Chan's. This may probably be attributed to the differences in intrinsic activity since the specific activities of our starting materials are much lower than that of Chan. When compared with Chan's results, the present method seems to be better not only because of the higher yield with larger extent of purification but also it was less time-consuming.

PART II. CHARACTERIZATION STUDIES

1. Analytical Polyacrylamide Gel Electrophoresis

Disc electrophoresis of our final products (MT-D-2 and MU-D-2) showed one but diffused band (Fig. 5). In the studies of other authors, diffused bands were also observed in the polyacrylamide gel electrophoretograms of U-HCG (Canfield et al., 1971; Ashitaka et al., 1970) and molar HCGs (Pala et al., 1972; Ashitaka et al., 1972) It has been suggested by several authors that there appears to be considerable heterogeneity among the HCG molecules. This heterogeneity principally exist in the carbohydrate portion of the molecule, and evidence for variation in sialic acid content has been discussed (Bell et al., 1969; Van Hall et al., 1968). The resultant heterogeneity in electrical charge has led to either broad or multiple bands in polyacrylamide disc gel electrophoresis.

As can be seen from the electrophoretograms in Figs. 5 and 6, the HCG band of U-HCG ran faster than those of MU-HCG and MT-HCG. This is consistent with our results on sialic acid determination (Table 10) that molar HCGs have lower sialic content than U-HCG (Bahl, 1969a).

2. N-terminal Amino Acid Determination

Two major N-terminal amino acids, serine and alanine,

were found in MT-HCG. Besides, small amount of valine together with traces of aspartic or glutamic acid were also observed. The findings in MU-HCG were similar to those of MT-HCG.

The amino acid sequence of U-HCG determined by Bellisario et al. (1973) and Morgan et al. (1975) revealed that the N-terminal amino acids for the α-subunit is alanine and for the β is serine. Morgan et al. (1975) found that approximate 10 and 30% of the α-subunits lack the initial 2 and 3 N-terminal amino acid sequence is: Ala-Pro-Asp-Val-Glu-Asp- (Morgan et al., 1975), certain amounts of aspartic acid and valine should exist as terminal amino acids. These findings are in good agreement with our results for MT-HCG and MU-HCG.

3. Immunological Studies (Double Immunodiffusion and Immunoelectrophoresis).

Immunodiffusion and immunoelectrophoresis of commercial U-HCG against absorbed anti-U-HCG antiserum demonstrated the existence of several precipitin lines in addition to the HCG specific line (Fig. 8 and 9). These extraneous antigen-antibody precipitin lines may be attributed to:

a. the presence in commercial U-HCG of non-HCG antigens which are unique to pregnancy (Hamashige and Arquilla, 1963 and 1964),

b. the presence of HCG subunits in commercial U-HCG (Vaitukaitis, 1974; Vaitukaitis et al., 1976) and

c. the incomplete removal of non-pregnant urinary protein specific antibodies by absorption.

Since the absorbed antiserum developed no precipitin lines against urinary proteins and human serum albumin on immunoelectrophoresis and immunodiffusion, the last possibility can be eliminated. Our final products, MT-D-2 and MU-D-2, showed only one precipitin line respectively when studied by immunoelectrophoresis and immunodiffusion (Figs. 8 and 9). In immunodiffusion (Fig. 8B), these lines fused completely with each other and with the inner line of commercial U-HCG without any spur detected. Based on this observation, it appears that the three HCGs obtained from different sources are immunologically identical. The inner band arised from commercial U-HCG is simply derived from the native HCG and its specific antibody, while the outer bands are attributed to the precipitation reactions between the extraneous antigens and their specific antibodies.

Double immunodiffusion patterns in Figs. 8A, C and D clearly show that none of the fractions of molar tissue contain the non-HCG components as mentioned above. This is confirmed by the presence of only one precipitin line in immunoelectrophoretic study of MT-2MTCA and MT-D-2 (Fig. 9). However, as can be seen from the immunodiffu-

sion patterns of MU-2MTCA in Figs. 8A and E, small amount of the extraneous antigens was found in this fraction. Immunoelectrophoresis of MU-2MTCA also revealed some additional precipitin lines, but the number was less than that of commercial U-HCG (Fig. 9). These findings can be reasoned by the previous reports on the presence of pregnancy unique antigens and the absence of HCG subunits in molar urine extracts (Hamashige et al., 1966; Vaitukaitis et al., 1976; Vaitukaitis and Ebersole, 1976). In view of the immunodiffusion patterns derived from MU-D-1, MU-D-2, MU-D-3 and MU-D-4 (Fig. 8E), it is clear that the extraneous antigens in MU-2MTCA can be effectively isolated by DEAE-Sephadex A-50 chromatography and become the major components in MU-D-3 and MU-D-4.

Since the MT-$MgCl_2$, MT-1MTCA and MU-$MgCl_2$ fractions which were eluted from the affinity column with less drastic eluates developed no precipitin lines in their immunodiffusion patterns (Figs. 8C and E), the components in these fractions may be attributed to the proteins and impurities nonspecifically bound to the affinity column (Murphy, 1974). The observation of diffused precipitin lines which cross-react with the HCG line for MU-1MTCA and MT-D-4 (Figs. 8D and E) probably suggested the existence of degraded HCG (Canfield et al., 1971) or antigens that have similar HCG-like determinants in these fractions.

When the results of immunodiffusion are viewed in con-

junction with those of polyacrylamide gel electrophoresis, two major problems we encountered in affinity chromatography are revealed. One of the difficulties is the non-specific adsorption of proteins to the affinity column. This adsorption was found to be largely due to non-bio-specific ionic and hydrophoblic interactions (O'Carra, 1974). The other arises from the specific binding to the affinity column of the pregnancy significant antigens which exist in molar urine. These problems have led to the necessity of further purification of MU-2MTCA and MT-2MTCA by DEAE-Sephadex A-50 chromatography. On the other hand, since MU-2MTCA contains more non-HCG contaminants which are largely due to the existence of the pregnant significant antigens, it is not surprising that MU-2MTCA was found to have lower HCG specific activity than that of MT-2MTCA

Since the above immunodiffusion results show that U-HCG, MU-HCG and MT-HCG have identical immunological property and the carbohydrate part of HCG has been suggested not to act as antigenic determinants (Bahl and März, 1974), the three HCGs may be expected to have similar amino acid composition (Table 5).

4. Molecular Weight Determination

Previous estimations of the molecular weights of highly purified U-HCG preparations as reported by various investigators differ quite widely according to the method

used. Values as small as 38,000 and greater than 67,000 have been reported by Mori (1970) and Canfield et al. (1971) respectively. More recently, Carlsen et al. (1973), based on the HCG primary structure, calculated a value of 37,900 with 14,900 for the α-subunit and 23,000 for the β-subunit.

The molecular weight of the purificied HCG obtained from urine, plasma and chorionic tissue of molar patient was shown by SDS electrophoresis to be 63,000, with 27,000 and 35,000 for the α and β subunits respectively by Pala et al. (1973). Ashitaka et al. (1972) postulated that HCG molecules from molar source, having much larger amount of sugar component and being eluted earlier on Sephadex gel filtration, should be larger than that of U-HCG.

In the present SDS electrophoresis, MU-HCG, MT-HCG and commercial U-HCG were dissociated by 2-mercaptoethanol into two components migrating with different molecular weight characteristics (Fig. 10). The faster and slower components, corresponding to molecular weights of 20,000 and 31,000 respectively, were readily identified to be the α and β subunits. No differences were observed in the components obtained from three different sources. It is obvious that our results are smaller than those of Pala et al. (1973) as well as those predicted by Ashitaka et al. (1972). However, they are in the same range as those obtained from U-HCG by Canfield et al. (1971), by a

similar method.

5. Optical Rotatory Studies

As the ORD curves for MU-HCG and MT-HCG are practically identical, it appears that they have a similar overall conformation. Treatment of the ORD data by the Moffitt equation indicates the absence of α-helical content. Since proline structurally prohibits the formation of α-helix in the vicinity along the peptide chain, very little α-helical structure in both HCG sample can also be expected in the light of the unusually high proline content in their amino acid compositions (Table 5).

By circular dichroistic measurements of U-HCG and asialo-U-HCG, Mori and Hollands (1971) concluded that HCG and its asialo derivative is practically devoid of the α-helical conformation. These authors have also remarked that both HCG and asialo-HCG are compact, nearly spherical molecules and they have very similar conformation. On the other hand, Hilgenfedt et al. (1972) have shown that the six HCG constituents isolated by isoelectric focusing from highly purified U-HCG have the same conformation and they have little, if any, α-helix. These results probably imply that sialic acid plays a very minor role in the HCG conformation.

6. Absorption Spectrum and Extinction Coefficient

As shown in Figs. 12A and B, both HCG preparations display an absorption maximum at 278nm. The extinction coefficients, $E^{1\%}_{1cm\ 280nm}$, were determined to be 4.93 and 4.86 for MT-HCG amd MU-HCG respectively. These results probably indicate a similar composition of aromatic amino acids in the two HCG preparations (see 'Amino Acid Composition' in Table 5).

The extinction coefficient of U-HCG has been reported by several authors. Mori and Hollands (1971) obtained a value of 5.5 which is higher than that of Morgan et al. (1974) 3.6; and that of Bahl (1969), 3.88 . In view of this large variation in the reported results on U-HCG, it is still not the time to compare at this point between U-HCG and HCGs from molar source.

7. Amino Acid Composition

Table 11 listed the amino acid compositions of various HCG preparations. Our results show that MU-HCG and MT-HCG have nearly the same amino acid composition. Except with lesser amounts of arginine and half-cystine, they are in good agreement with those of U-HCG (Bahl, 1969a) and HCG from male choriocarcinoma urine (Canfield et al., 1971). Though HCGs from different sources have thought to be chemically different, many of the differences noted might

Table 11. Amino Acid Compositions of HCGs from various Sources[a]:

Amino Acid	MT-HCG	MU-HCG	U-HCG[b]	CU-HCG[c]
Lysine	9.9	10.0	9.3	10.1
Histidine	4.0	4.0	4.0	4.0
Arginine	10.6	10.6	14.2	14.3
Aspartic Acid	16.6	16.9	16.1	15.6
Threoine	16.7	16.8	15.6	15.8
Serine	16.5	16.8	17.9	17.9
Glutamic Acid	19.2	19.3	16.8	17.1
Proline	25.7	25.6	26.3	27.4
Glycine	12.6	13.3	11.6	9.7
Alanine	11.3	12.2	11.6	10.9
Half Cystine	12.8	11.1	18.5	17.7
Valine	16.7	17.0	15.9	16.2
Methionine	3.7	3.5	3.6	3.6
Isoleucine	6.4	6.3	5.1	5.5
Leucine	15.5	15.6	13.7	13.7
Tyrosine	6.0	6.3	5.9	5.7
Phenylalanine	7.3	7.6	5.2	5.3

[a] Results were calculated on the basis of 4 histidine residues. No corrections have been made for destruction during acid hydrolysis.

[b] Bahl's data (1969).

[c] HCG from male choriocarcinoma urine (Canfield et al., 1971).

be accounted for by variations in the carbohydrate structures. Their amino acid compositions have been found to have no significant difference (Canfield et al., 1971; Ashitaka et al., 1972).

8. Carbohydrate Composition

The carbohydrate compositions of our molar HCG preparations are listed in Table 12 together with the data various HCGs from different authors. As can be seen, the carbohydrate compositions of our two HCGs obtained from molar tissue and molar urine are quite similar but less than those of various HCGs reported by different investigators. In our preparations, galactose was found to exist in larger amount than mannose. This finding is consistent with the observation of Ashitaka et al. (1972) that HCGs originated from hydatidiform mole may have more galactose than mannose. In addition, low amino sugar content (3.3%) in MT-HCG was also found by the same group of authors. The relatively smaller amount of sialic acid in our products than that of U-HCG (Bahl, 1969a) has been revealed by their different mobilities in polyacrylamide gel electrophoresis as discussed earlier (p. 74).

As it has been suggested that the chemical differences in HCGs between trophoblastic diseases and normal pregnancy are mainly due to their variations in carbohydrates (Canfield et al., 1971; Ashitaka et al., 1972). Our data also show,

Table 12. Carbohydrate Composition of HCG from Various Source (gm/ 100gm).

Carbohydrate	MU-HCG	MT-HCG		Placental-HCG (1st trimester)	U-HCG
	Our Preparation	Our Preparation	Ashitaka et al.* (1972) LH-like	Ashitaka et al. (1970)	Bahl (1969)
Fucose	±	±	+++	+++	0.6
Galactose	6.7	5.5	+++	+++	5.3
Mannose	2.1	2.3			5.3
Total Hexose	8.8	7.8	40.0	23.0	11.2
N-acetyl-galactosamine	±	±	+++	+++	2.2
N-acetyl-glucosamine	5.4	4.8	+++	+++	8.9
Total Hexosamine	5.4	4.8	3.3	8.0	11.1
Sialic Acid	6.1	6.2	8.2	3.0	9.0
Total Carbohydrate	20.3	18.8	51.5	34.3	31.3

* Determined by colorimetric methods.

± Signifies present in small amount.

+++ Not determined.

when compared with those contained in the above mentioned references, similar discrepancies in the sugar moiety, i.e. more galactose than mannose present in molar HCGs.. This finding appears to further support their postulation.

In summary, our results by means of acrylamide gel electrophoresis, immunodiffusion, immunoelectrophoresis, molecular weight and N-terminal amino acid determinations support the hypothesis that two highly purified HCG preparations can be obtained from molar tissue and molar urine by the procedures described in the present study. Further characterization of the preparations indicates that MU-HCG and MT-HCG are physically and chemically similar. However, molar HCGs possess different chemical and physical profile from normal pregnant HCGs; we consider that such discrepancy is due mainly to the carbohydrate moiety of the HCG molecules.

SUMMARY

Highly purified HCG preparations were obtained from urine and trophoblastic tissue of patients with hydatidiform mole by 3 fractionation steps:

a) Acid and salt precipitation;

b) Immunoaffinity chromatography; and

c) DEAE-Sephadex A-50 chromatography.

The total HCG activities recovered from molar tissue and molar urine were 27% and 22% respectively. The purity of the HCG preparations were examined by disc and immunoelectrophoresis, immunodiffusion, and N-terminal amino acid determination. When determined by SDS electrophoresis, both MU-HCG and MT-HCG shown a molecular weight of 51,000, with 20,000 and 31,000 respectively for the α and β subunits. The amino acid and carbohydrate composition, α-helical content and extinction coefficient of each HCG preparation were also determined. It appears that no significant differences in physical, chemical and immunological properties between MU-HCG and MT-HCG were observed in the present study.

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